THE INFLUENCE OF DRUG USE AND APOLIPOPROTEIN E ON HIV RELATED DISEASE OF THE CENTRAL NERVOUS SYSTEM

Juan Carlos Arango-Viana

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DECLARATION

I declare that all the work presented in this thesis is of my own composition, and the studies described were undertaken by myself.

Juan Carlos Arango-Viana

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AANATF	American Academy of Neurology and AIDS task force
AD	Alzheimer's disease
AICD	Activation induced cell death
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APPK	Kunitz-containing amyloid precursor protein
ATP	Adenosine triphosphate
AZT	Zidovudine
BBB	Blood brain barrier
CA	Capside Antigen
CAA	Cerebral amyloid angiopathy
CD	Cluster determinant
CDC	Centers of disease control
CDSC	Communicable disease report
CEPT	Cholesteryl ester transfer protein
CJD	Creutzfeldt-Jakobs disease
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebro spinal fluid
CTL	Cytotoxic lymphocyte
DD	Death domain
DED	Death effector domain
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DOR	Delta opioid receptor
DU	Drug users
ELISA	Enzyme-linked immunoabsorbent assay
Env	The virus envelope

ELISA	Enzyme-linked immunoabsorbent assay
Env	The virus envelope
EU	Exposed uninfected
FADD	Fas associated protein with death domain
FasL	Fas ligand
FDC	Follicular dendritic cell
FLICE	FADD-like ICE inhibitory protein
Gag	Group specific antigen
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony stimulating factor
GCTR	G protein coupled transmembrane receptor
HAD	HIV associated dementia
HAART	Highly active antiretroviral therapy
H and E	Haematoxilin and Eosin
HIV	Human immunodeficiency virus
HIVE	HIV encephalitis
HSPG	Heparan sulphate proteoglycan
HSV	Herpes simplex virus
ICE	Interleukin converting enzyme
IDL	Intermediate density lipoprotein
IDU	Intravenous drug use
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
KDa	Kilodalton
KOR	Kappa opioid receptor
LCAT	Lecitin cholesterol acyltranferase
LDL	Low density lipoprotein
LN	Lymph nodes
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide

LRP	Low density lipoprotein receptor
LTR	Long term repeat
MA	Matrix protein
MAPK	Mitogen-activated protein kinase
mbTNFα	Membrane bound tumour necrosis factor alpha
MCP-1	Monocyte chemotactic protein-1
MDC	Macrophage derived chemokine
MHC	Major histocompatibility complex
MIP 1 α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metaloproteinase
MNGC	Multinucleated giant cell
MOR	Mu opioid receptor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSM	Men who have sex with men
NC	Nucleocapside
Nef	Negative factor
NGF	Nerve growth factor
NK	Natural Killer
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NSI	Non-syncitium inducing
NtRTI	Nucleotide analogue reverse transcriptase inhibitor
PAF	Platelet activating factor
PAR-4	Prostatic apoptosis response 4
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PI	Protease inhibitor
PNS	Peripheral nervous system
RANTES	Regulated upon-activation normal T expressed and secreted
RAP	Receptor associated protein

Rev	Regulator viral expression
RRE	Rev response element
RT	Reverse Transcriptase
RTI	Reverse transcriptase inhibitors
SAAP	Secreted isoforms of amyloid precursor protein
SDF-1	Stromal derived factor 1
SHIV	Simian/human immunodeficiency syndrome
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
SLC	Secondary lymphoid tissue chemokine
SU	Surface
Tat	Transcriptional transactivator
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	T helper
TM	Transmembrane
TNFα	Tumour necrosis factor alpha
TNFαRII	Tumour necrosis factor alpha receptor II
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
UK	United Kingdom
UNAIDS	Joint united nations programme on HIV/AIDS
Vif	Virion infectivity factor
VLDL	Very low density lipoprotein
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
WB	Western Blot
WHO	World Health Organisation

ABSTRACT

Since June 5th 1981, when the first five cases of Acquired Immunodeficiency Syndrome (AIDS) were described, it is estimated that 22 million people have died from this disease worldwide. The causative agent of AIDS is the Human Immunodeficiency Virus (HIV). Injecting drug use and high-risk sexual behaviour are the main risk factors for contracting HIV infection. Moderately effective therapy is now available but that remains an expensive option. In 20-30% of untreated patients in late stage HIV infection, a cognitive and motor dysfunction known as HIV associated dementia (HAD) has been described and many of these patients have HIV encephalitis (HIVE) directly attributable to the virus. The severity of HIVE is variable but may be associated with severe brain damage. It is known from study of other diseases that the response to brain injury is somewhat dependent on the host Apolipoprotein E genotype.

Aims: I) To investigate the contribution of host factors such as drug use (DU) and apolipoprotein E (ApoE) genotype in the development of HIVE and HAD. II) To investigate some histological changes relevant to HAD in drug users with different ApoE genotypes.

Methods: Genotyping for ApoE was carried out in 312 individuals who were divided into five groups: Group I consisted of 64 normal controls (alive), Group II consisted of 82 HIV negative drug users (alive), Group III consisted of 38 (deceased) pre-symptomatic HIV positive drug users (pre-AIDS), Group IV consisted of 84 drug users with AIDS (deceased), Group V consisted of non-drug users with

AIDS,all deceased, 6 of these were haemophiliacs and 38 men who have sex with men (MSM). Published data for ApoE genotype in 400 individuals drawn from the general Scottish population were used for comparison (Group VI). When comparing the frequency of different ApoE alleles among different risk factor groups, the drug using groups (III and IV) were analysed together. Details of the clinical information including neuropsychological evaluation and CD4 and CD8 counts were extracted from patient databases.

Detailed histological examination was carried out in 49 cases on Group IV and 29 cases of groupV with particular emphasis on comparing cortical and subcortical grey matter. From the patient groups detailed above, a quantitative analysis of CD68 immunopositive cells (microglia and macrophages) was performed in three different areas of the brain including frontal lobe, temporal hippocampus and thalamus. Four cases were taken from Group I, ten from Group II, seven from group III, nine from Group IV and six from GroupV. Sections were analysed using a Leica Q500iw) analysis system. Cases were processed and analysed blind to group and ApoE genotype.

Results: Analysis showed that the frequency of ApoE *ε2* allele in the HIV positive drug users (Groups III and IV taken together, n=122) was 14.3%. In contrast, the frequency of the ApoE *ε2* in Groups I (n=64) and II (n=82) was 5.4% in each group and 8.2% in the normal Scottish population (Group VI, n: 400). These differences were significant (X^2=7.017, p<0.008, and X^2=7.580, p< 0.006, respectively). At the same time, the ApoE *ε3* allele for Groups III and IV was found to be under-represented when compared to the Scottish population (X^2=9.695, p<0.002). More detailed comparisons between different genotype subsets within the groups all gave

results supportive of the main finding of ApoE *ε2* over-representation. Group V showed no difference in ApoE genotype from the general population.

CD4 and CD8 counts were found to be significantly higher in Group IV compared with Group V. 61.5% of Group IV individuals had CD4 counts above 50th percentile, in contrast to only 28.5% in individuals in Group V (X^2=11.769, p<0.001). For CD4/CD8 ratios, 61.5% of the group IV cases had values above the 50th percentile, while only 26.3% of Group V were above (X^2=8.622, p<0.002). The ApoE *ε2* allele was found associated with higher CD8 counts (X^2=5.321, p<0.02). The histological change which was most strongly associated with dementia was HIVE (X^2=14.977, p<0.0005). The association of ApoE alleles with HIVE showed that ApoE *ε2* and *ε3* alleles were significantly associated with HIVE (X^2=4.007, p<0.05 and X^2=5.090, p<0.02 respectively), although the direction of the association with ApoE *ε3* allele was negative. Quantitative analysis of CD68 positive cells showed that Groups IV and V had higher levels of positivity than Groups I, II and III, in almost all areas studied. The differences were not significant when Group IV was compared with Group V. In the two groups, cases with ApoE 3\3 genotype tended to have lower values than cases with 3\2 or 3\4 ApoE genotype and this reached significance in some brain areas.

Conclusions: The ApoE genotype of HIV positive drug users differs from that of HIV negative drug users in a close knit Edinburgh population and from the normal Scottish population. An over-representation of ApoE *ε2* alleles and under-representation of the ApoE *ε3* alleles accounts for the difference. These two alleles have an opposite effect in the development of HIVE, and possibly in an indirect way in the establishment of HAD. This effect might be related to the reaction of CNS to

inflammatory insult, which appears to be less severe in individuals with the ApoE 3\3 genotype. The finding that activated microglial cells appear to be less numerous in association with ApoE 3\3 genotype supports this supposition.

GENERAL INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). Since June the 5th 1981, when the first five cases of AIDS were described (CDC, 1981), it is estimated that 22 million people have died of this disease. It is also estimated that 36 million people are infected with HIV worldwide (Piot et al, 2001).

Among the risk factors for contracting HIV infection are injecting drug use and high-risk sexual behavior.

Untreated HIV infection is associated with a number of conditions which result from the immune suppression caused by a significant decline in CD4+ T lymphocytes in late stages of the infection as well as a direct cytopathic effect of HIV.

In about 20-30% of patients in the late stages of HIV infection, a cognitive motor complex develops which is also known as HIV associated dementia (HAD). The pathogenesis of HAD is unclear, but inflammatory mediators have been suggested to play a significant role in the establishment of this condition (Achim et al, 1993; Griffin et al, 1994; Seilhean et al 1997; Yeung et al 1995; Wilt et al, 1995; Vitkovic et al, 1995; Nath et al, 1999; Persidsky et al, 2001). Many of the cases affected with HAD also have HIV encephalitis (HIVE) at histological examination, but in a number of HIVE cases the cognitive function is unimpaired. Conversely in many cognitively impaired cases, no

evidence of HIVE is found at histological examination. Absolute correlation between HIVE and HAD has not been documented and a number of factors may influence the development of both of these conditions. These include viral and/or host factors.

Different schemes have been developed for the treatment of HIV infection, but all (including highly active anti-retroviral therapy) have failed to completely eradicate the virus from infected patients (Chun et al, 1997a; Zhang et al, 1999). An increased incidence of neurological complications of HIV infection is expected, due to the poor penetration of antiretroviral drugs into the central nervous system (CNS) (Sacktor et al, 2001). In Edinburgh, a differential incidence of HIVE has been observed among cases with different risk factors for contracting HIV infection. Intravenous drug users have higher incidence of HIVE than cases that contracted the infection by sexual contact (Bell et al 1996, 1998). There is no apparent reason for the differential incidence on HIVE among cases with different risks for contracting HIV infection, but this finding suggests that host factors may be relevant for developing this condition.

Apolipoprotein E (ApoE) is a lipid transport protein and a major determinant in lipoprotein metabolism. Three ApoE isoforms exist and they are known as ApoE E2, E3 and E4. This polymorphism is the result of three alleles at a single gene locus.

In recent years, a growing body of evidence linking some ApoE isoforms with neurodegenerative conditions, differential outcome after acute brain insults and immune system functions have been published (Strittmatter et al, 1993; Sorbi et al, 1995; Sheng et al, 1998). Previous studies linking ApoE with the cognitive impairment associated

with HIV infection have shown apparently contradictory results. While in one the *ε4* allele of ApoE was found significantly associated with cognitive impairment (Corder et al, 1998), in the other no evidence of this was found (Dunlop et al, 1997).

The present study aimed to:

- Investigate the contribution of drug use and ApoE genotype in the development of HIVE and HAD.
- Investigate some histological changes relevant to HAD in drug users with different ApoE genotypes.

To achieve these aims, five groups of patients were studied. Group I included normal control cases, Group II consisted of HIV negative drug users, Group III pre-symptomatic HIV positive drug users, Group IV consisted of HIV positive drug users with AIDS and Group V consisted of non-drug users with AIDS (mainly men who have sex with men (MSM)).

All the study population was genotyped for ApoE using fresh tissue, in the great majority of cases. Histological examination of a number of HIV positive drug user and HIV positive non-drug user cases was also carried out. Some histological features were compared between the groups and among different ApoE genotypes.

Relevant clinical information for HIV positive cases such as cognitive status and the last CD4/CD8+ T cell counts were extracted from the Edinburgh brain data bank.

The number of microglial cells in white and grey matter of three different areas of the brain was estimated by computerized image processing in a selected number of cases of each group.

For the statistical analysis, Chi square method was selected for comparisons of ApoE genotypes among different groups and for the comparisons of clinical and histological changes. Analysis of variance with Tukey post-Hoc tests were used for comparing the number of microglial cells in different brain areas among the five groups studied.

Chapter 1

HIV AND AIDS

1.1 Introduction

The Acquired Immune Deficiency Syndrome (AIDS), is caused by infection with Human Immunodeficiency virus (HIV) type 1 or 2 (HIV-1 or HIV-2). These viruses are neurotropic retroviruses, which belong to the Lentivirinae (Lentivirus) subgroup. The term Lentivirinae was first used in 1954 to describe causative agents of certain infectious diseases affecting sheep. Viruses belonging to this sub-family are neurotropic and can also infect cells of the monocyte/macrophage lineage. One of the most important characteristics of diseases associated with this sub-family is the long latent period before occurrence of disease. Lentiviruses can cause immune deficiency in other non-primate species such as felines and bovines. These viruses are consequently known as feline immunodeficiency virus and bovine immunodeficiency virus. Primates can be infected by HIV-1 and HIV-2 in the case of human primates or by simian immunodeficiency virus (SIV) in the case of non-human primates. There is 75% genetic homology between SIV and HIV-2, and both viruses have a similar geographic distribution, mainly found in certain western and central African regions. For these reasons it has been hypothesized that HIV-2 was transmitted from monkeys to humans. HIV-2 infection was first described in 1985 in west Africa and since then cases have been reported in central Africa, Canada, western Europe, Brazil and America. By 1989, six cases had been reported to the

centers of disease control (CDC) in America and all of them were imported cases (CDC, 1989). HIV-2 seems to be largely restricted to Africa.

The first reports of AIDS date back to 1981 (CDC, 1981) but it took another two years to recognize the central nervous system (CNS) involvement. In the late eighties, large pathological studies in America and Europe disclosed neuropathological abnormalities in 80% and 90% of the cases respectively (Everall and Lantos, 1991).

Lentiviruses can cause disease by direct invasion of cells and tissues and in an indirect way by favoring opportunistic conditions to arise after the immune deficiency is established (reviewed in Atwood et al, 1993).

Transmission of HIV from person to person can occur through contact with infected body fluids, (especially those rich in inflammatory cells such as blood, semen, breast milk), through mucosal surfaces, broken skin and through intravenous exposure. Vertical transmission from mother to child is still a problem in developing countries in which highly active anti-retroviral therapy (HAART) is not widely available. In such countries, one third of the mother to child transmission is due to breast-feeding and the remaining two thirds are transmitted in-utero or at time of delivery. In developed countries, HAART was introduced in mid 1990s; this treatment regimen consists of two reverse transcriptase inhibitors (RTI) plus one protease inhibitor (PI) (see later) and since its introduction it has impacted decisively the vertical transmission of HIV. In developing countries, Zidovudine (AZT) a RTI monotherapy, is still widely used and even though, it has been shown to significantly lower the risk of vertical transmission (CDC, 1998), resistant viral strains emerge

rapidly (see later). This accounts for the high rate of vertical transmission of HIV observed in those countries.

There are several major risk factors for contracting HIV infection. These include:

Men who have sex with men (MSM), the leading cause of new infections in America.

Heterosexual exposure, the most important risk factor for contracting the infection in women and the second in importance in America.

Intravenous drug use (IDU), the third most important risk factor, accounting for almost a fifth of the new cases in America.

Maternal transmission, the most important cause of paediatric AIDS.

Blood or blood product transfusion is still a risk factor worldwide.

In the United Kingdom the highest HIV prevalence is found among MSM and a substantial level of transmission has been suggested among people with this risk factor. A rise in HIV prevalence for women in London have also been observed, suggesting that heterosexual transmission of HIV has risen (CDSC, 1999). Men are more often infected worldwide than women, except in sub-Saharan African countries where the situation is the reverse. In America (USA) 70% of new adult cases reported to the CDC in 1999 were men. Among men, MSM is the leading cause accounting for 60% of all new cases. IDU was found in 25% of new cases, while only 15% of new cases were due to heterosexual exposure. In women on the other hand, heterosexual exposure accounted for 75% of all new cases, African American women being the most affected. Consequently two thirds of the pediatric cases reported in 1999 to the CDC are African American (CDC, 2001).

In 1988, 69,000 AIDS cases had been reported to the CDC in America and 108,000 AIDS cases were reported world-wide (Janssen et al, 1989). By 1989, AIDS was the leading cause of death in men between 25-44 years old and women between 25-34 years old in New York city (Janssen et al, 1989), and in 1993 it was the leading cause of death in both young men and women in Spain (Castilla et al, 1997).

By 1999 official reports accounted for 33.4 million people living with HIV/AIDS worldwide of which 22,5 million were in Africa alone (for a review see Schwartz and Nair 1999; Satcher, 1999). In America an increase in number of AIDS cases, from 270.000 in 1997 to 317.000 in 1999 has been observed. Metropolitan areas have the greatest prevalence of AIDS cases (83%), and men account for 79% of all cases, most of the affected patients are African Americans and White Americans. Most AIDS cases (77%) have been observed in people between 35 and 64 years of age. The number of adolescents and children living with AIDS in America at the end of 1999 was 4,300 (CDC, 2001).

By the end of the year 2000 it was estimated that 21.8 million people had died of AIDS since the beginning of the epidemic. Of those, 4.3 million were children. Mortality from HIV/AIDS among men and women varies considerably from country to country (Heath et al, 1998). Newly infected people in the year 2000 alone numbered 5.3 million and the total number of people living with HIV/AIDS worldwide rose to 36.1 million. 3 million deaths worldwide were caused by AIDS. In the UK it was estimated that there were 31,000 people living with HIV/AIDS at the beginning of the year 2001 (UNAIDS, 2000).

The increase in the prevalence of AIDS may in part be due to an overall increase in survival following the introduction of HAART. In the first half of 1996, a 23%

reduction in deaths among AIDS cases was officially published by the American CDC (CDC, 1997). European figures have also shown a slowing in HIV disease progression in homosexual men (Hendriks et al, 2000). This is probably due to the availability of HAART, which has produced an effect similar to the use of prophylaxis against Pneumocystis Carinii pneumonia (which also slowed the progression to AIDS in the early nineties) (Longini et al, 1993). No geographical variation in HIV disease progression was observed in European intravenous drug users, before HAART became available (Prins et al, 1999).

1.2. Human Immunodeficiency Virus (HIV)

HIV is an RNA virus of the lentiviruses subgroup of retroviruses. The HIV genome consists of nine different genes divided into two genes for structural protein (*gag* and *env)*, one gene (*pol*) for viral enzymes, two genes for activation of HIV gene expression (*tat* and *rev*) and four genes for accessory factors (*vif, vpu, vpr* and *nev*). These nine genes are contained between the 5' and 3' long terminal repeat regions (LTR). The 5' LTR regulates the initiation of RNA transcription and the 3' LTR the termination of the transcription.

1.2.1. HIV genetic organisation

The genes and gene products of the HIV virion are displayed in table 1.1.

The *gag* (group specific antigen) encodes a 55 kilodalton (Kda) precursor protein known as Pr 55 ^{gag}. This precursor protein is cleaved by the viral protease (one of the enzymes encoded by *pol* gene) into four smaller proteins. These proteins are:

The nucleocapsid, (NC) is a 9 KDa (p9) protein and acts as an RNA binding protein.

The capsid (CA) which is 24KDa (p24).

A 6KDa protein (p6) which regulates viral release from the cell.

Matrix protein (MA) which consists of a 17KDa protein located between the capsid and envelope.

The capsid (CA) is a cone-shaped structure, which holds the two 9.2 KDa strands of RNA, the viral enzymes, reverse transcriptase (RT), the integrase (IN) and the protease (all encoded by the *pol* gene), the NC and p7. The capsid is surrounded by MA protein and the viral envelope contains all these.

The envelope consists of a lipid bilayer, derived from the host, and two viral envelope glycoproteins known as gp120 or surface (SU), and gp41 or transmembrane (TM) proteins. These glycoproteins are produced by the cleavage of a 160 KDa (gp160) protein by a cellular protease in the Golgi apparatus of the cell (for a review see Buck and Siliciano, 1996). The HIV gp120 regulates viral entry to the host cell and HIV tropism. This, gp120 is subdivided into five different variable loops (hypervariable region) termed V1-V5 and five conserved regions (C1-C5). Loops V1 and V2 regulate viral entry and together with V4 and V5 mediate interaction with gp41. V3 is the major determinant of viral tropism (Reviewed in Ratner 1996).

The HIV gp41 consists of three main domains, the ectodomain, the membrane spanning domain and the carboxyl-terminus. The fusion event is mediated by the hydrophobic amino-terminus domain. The carboxyl-terminus domain contains two amphipathic helices, which may contribute to some of the cytopathic effects of the virus (reviewed in Ratner, 1996).

Table 1.1. Genes and gene products encoded by the HIV genome

Gene/s	Protein Type	Size of protein	Cleavage Products
Gag	Structural	55Kda,	p17(MA), p24 (CA)
		$Pr55^{gag}$	p9 (NC), p6
Env		160Kda	gp120, (SU)
		gp160	gp41 (TM)
Pol	Enzymatic	180 Kda	Protease
			RT
			Integrase (IN)
Tat	Regulatory	16 Kda	
Rev		13 Kda	
Vif	Accessory	23 Kda	
Vpu		18 Kda	
vpr (HIV-1)		16 Kda	
vpx (HIV-2)		16 Kda	
Nef		27-34 Kda	

Key: p6: protein 6, p9: protein 9, p24: protein 24, NC: nucleocapsid, MA: matrix protein, CA: capsid protein, SU: surface protein, TM: transmembrane, RT: reverse transcriptase, IN: integrase, gp: glycoprotein, Pr 55^{gag}:Gag precursor protein, KDa: kilo Dalton.

1.2.2. Regulatory proteins

Tat.

The HIV transcriptional activator (Tat), is a 16 KDa transcriptional transactivator protein, which is encoded by the (*tat*) gene. This protein mediates its actions through

the Tat response element (TAR), which is present in the viral RNA. The function of Tat is to enhance the overall expression of HIV RNA expression. In other words, Tat enhances viral replication (for review see Ratner, 1996; Atwood et al, 1993).

The HIV virus, unlike oncoviruses, does not require cell division in order to incorporate its genetic material into the genome of the host. This is achieved by the formation of the so-called pre-integration complex (PIC) which directs the viral genome into the nucleus by accessing intracellular transport pathways. The PIC consists of two strands of reversed transcribed DNA from the viral RNA template (mediated by RT), the IN enzyme, the Gag MA and one accessory protein known as viral protein R (Vpr). The pre-integration complex is formed soon after the uncoating of the viral capsid, which follows the fusion of HIV with the plasma membrane of the host. It has been demonstrated that activated CD4+ T cells expressing the appropriate chemokine receptor (see later) are permissive for HIV productive infection. Cytokines can mediate this T cell activation in a wide variety of lymphoid phenotypes (Unutmaz et al, 1999).

Other actions of Tat include the transactivation of some cellular genes such as those for interleukin (IL) 1 and IL 6 and the genes of other viruses.

Tat enhances HIV replication through the TAR element in the viral RNA by assisting in recruitment and activation of cellular factors that promote elongation activity of RNA polymerase II. A second action of Tat is to facilitate the spread of the virus by enhancing the expression of cytokine genes in the G2 phase of the cell cycle in a TAR independent fashion (Kashanchi et al, 2000). Tat also impairs IL12 production, which may decrease the activity of natural killer (NK) cells (for review see Rubartelli et al, 1998).

Rev

Rev is a post-transcriptional transactivator and enhances production of virion components. Rev is a 13KDa protein, encoded by the regulator of viral expression (*rev*) gene. This protein stabilizes viral mRNA. Rev binds to the Rev response element (RRE) in incompletely and unspliced viral mRNAs, stabilizes them and mediates their export from the nucleus to the cytoplasm for translocation. As a consequence of this stabilization a shift from multiply spliced mRNA to incompletely spliced and unspliced mRNAs occurs (for review see Ratner, 1996; Atwood et al, 1993). Unspliced mRNA encodes viral structural and enzymatic proteins, while single spliced mRNA encodes envelope and accessory proteins.

1.2.3. Accessory proteins

This group of proteins are, at least partially, required for HIV replication. They include:

Virion infectivity factor (Vif)

Vif is a 23KDa protein encoded by *vif* gene. It is found in almost all lentiviruses and it functions by altering the structure of a virus particle, increasing its infectivity probably mediating an efficient proteolytic cleavage of structural and enzymatic precursor proteins. This protein is localized in the cytoplasm and plasma membranes of infected cells (for a review see Ratner, 1996).

Viral protein U (Vpu)

Vpu is an 18 KDa protein which is encoded by the *vpu* gene and is found in HIV-1 and Chimpanzee SIV, but not in other SIV or HIV-2 viruses. It is located in the cellular membranes within the lipid bilayer. Vpu has two different actions. It down-

regulates CD4 receptors by decreasing the CD4 stability at the level of endoplasmic reticulum and promoting the processing and transport to the cell surface of envelope glycoproteins. The other action of Vpu is related to viral release from the plasma membrane. Vpu enhances the release of viral particles from the infected cell. A proposed mechanism for this activity includes an ion channel activity of the N terminal domain of this protein (for a review see Ratner, 1996).

Viral protein R (Vpr) and Viral protein X (Vpx).

These proteins are encoded by *vpr* and *vpx* genes respectively. Vpr is found in all primate lentiviruses. Vpx is also found in some primate lentiviruses with the notable exception of the HIV-1. Vpx is a 16KDa cytoplasmatic protein and its function is not fully understood. In contrast, the 16 KDa Vpr protein is located in the cellular nucleus; forms part of the pre-integration complex and mediates the transport of this complex to the nucleus. Vpr actions are well studied and include the arrest of cells at the G2 phase of the cell cycle. Other activities are the enhancement of HIV-1 replication in macrophages and lymphocytes, co-activation of glucocorticoid receptor and other hormonal steroid receptors in the cellular nuclei (Kino et al, 1999) and the enhancement of HIV-1 long term repeat (LTR) activity. LTR is located at the 5' and 3' ends of viral RNA. Near the 5' end, TAR is found. Binding sites for host transcription factors are also located in the LTR. Vpr is also found circulating at detectable levels in HIV infected people, suggesting a possible paracrine action probably due to its glucocorticoid receptor action (Kino et al, 1999).

Negative factor (Nef)

This is a 27-34 KDa protein and is encoded by *nef* gene. It has been found in cellular membranes, cytoplasm, cytoskeleton and nucleus. It has a number of different

activities including the down-regulation of CD4, and major histocompatibility complex class I (MHC I), by different mechanisms (Akari et al, 2000), making infected cells less vulnerable to cytotoxic T lymphocyte (CTL) mediated lysis. Other Nef functions include enhancement of HIV infectivity and reverse transcription. In the virion, Nef is found to be associated with the viral core suggesting early activity during the viral life cycle (Kotov et al, 1999).

1.2.4. Replication Cycle

Several steps are required for the release of virions from infected cells. The process starts with viral attachment and entry into susceptible cells. This step requires the presence of at least two cell surface receptors namely CD4 and chemokine receptors (also known as HIV co-receptors).

In the extracellular amino-terminus domain of CD4 receptor is where the gp120 binding and major histocompatibility (MHC) class II binding sites reside (reviewed in Bowers et al, 1997 and Berger et al, 1999). A conformational change in gp120 occurs upon binding to CD4 receptor and exposes the co-receptor binding determinants on gp120. Then interaction between gp120 and the appropriate co-receptor is followed by further conformational changes freeing the previously buried fusion peptide in gp41 exposing its hydrophobic amino-terminus fusogenic domain. The fusogenic domain is inserted into the cell membrane of susceptible cells leading to membrane fusion (reviewed in Berger et al, 1999). Some CD4 independent gp120/co-receptor interactions have been described too (reviewed in Berger et al, 1999).

Chemokine receptors are small molecules, which belong to the seven transmembrane family of G protein-coupled receptors. They control lymphocyte migration and extravasation. Over 40 different chemokine receptors have been described (for a review see Zlotnik and Yoshie, 2000; Murphy et al, 2000; Mahalingam and Karupiah, 1999).

Chemokines have been divided into two major sub-families, based on the position of the two cysteine aminoacids of their N terminal fragment. In CC chemokines both cysteine residues are adjacent while in CXC chemokines, an aminoacid is located between them. Other classes of chemokines have also been identified, the C chemokine and CX3C which is also known as franctalkine. In the latter, three aminoacids are found between the two cysteine residues.

Chemokine receptors are subsequently designated as the sub-family of chemokine followed by the letter R. Thus there are nine different ligands or receptors for CC chemokines and they are designated CCR1 to 9. There are only five CXC receptors also designated CXCR1 to 5. The above-mentioned chemokine subclasses only have one ligand each. As previously mentioned, HIV-1 needs, apart from the CD4 receptors, an appropriate co-receptor. This HIV-1 co-receptor belongs to the chemokine receptor family. Early *in vitro* studies showed that non-human cells expressing human CD4 receptors were not infected by HIV, although membrane fusion was observed (Ashorn et al, 1990). Some human cell lines (different from lymphoid or cervical carcinoma cell lines) failed to be infected by HIV even when they were made to express CD4 receptors. This suggested that other cell surface molecules were required for efficient HIV infection (Chesebro et al, 1990).

Subsequent studies demonstrated a different tropism of HIV. Some HIV strains infected T lymphocytes (HIV T cell tropic, syncytium inducing -SI-), while others infected macrophages, also known as HIV M tropic non-syncytium inducing –NSI-. The hypervariable V3 loop of the gp120 was identified as the key element governing the cell tropism of HIV (De Jong et al, 1992a, 1992b; Fouchier et al, 1992).

The discovery of HIV co-receptors by different groups in 1996 explained both the lack of infectivity of HIV to non-human CD4 expressing cell lines and certain phenotypic variations in HIV tropism (Deng et al, 1996; Dragic et al, 1996; Feng et al, 1996; Alkhatib et al, 1996; Samson et al, 1996). This important discovery allowed the phenotypic HIV classification currently in use. It is now known that M-tropic, Non-syncytium-inducing (NSI) HIV use the CCR5 co-receptor (R5 virus) and the T-cell tropic, syncytium inducing (SI) HIV use the CXCR4 co-receptors (X4 virus). Furthermore, M tropic and T cell tropic variants, so-called dual tropic HIV (R5X4), have been discovered (Doranz et al, 1996).

The first discovered co-receptor was called fusin. It was shown to act as a co-receptor for T cell tropic or syncytium forming (SI) HIV-1 strains. Fusin was found to be identical to the α chemokine receptor CXCR4, which in turn, is the receptor for stromal derived factor 1 (SDF-1). This chemokine can effectively block CXCR4 receptors for HIV 1 fusion and entry into CD4+ T cells. The β chemokine regulated upon activation normal T expressed and secreted (RANTES), and macrophage inflammatory protein 1α (MIP 1α) and MIP 1β are the normal ligands for CCR5. Consequently, when these chemokines are bound to CCR5, it is phosphorylated and internalized and in that manner cannot be used as HIV-1 co-receptor to gain entry to

macrophages. More over, RANTES, is a very active inhibitor of HIV replication. Absence of this receptor (CCR5) is associated with resistance to HIV-1 infection.

There are lines of evidence supporting biochemical changes in the co-receptor molecule, accounting for the tropism of HIV-1 strains. Thus macrophages also express CXCR4 receptors and can be infected though less effectively than T lymphocytes, by SI or T cell tropic HIV 1 strains. (Lapham et al, 1999). Molecular studies in monocyte and macrophage surface receptors from different human donors have demonstrated differences in densities of both CD4 receptors and CXCR4/CCR5. This suggests that appropriate density of CD4 and co-receptors are important in regulating the HIV-1 fusion and entry into the target cell (Lapham et al, 1999).

Infected macrophages and monocytes also produce an excess of certain chemokines such as MIP1α and MIP1β, which in turn are chemo-attractants for non-infected cells (Schmidtmayerova et al, 1996). Viral replication and TNFα increase production of MIPα and MIPβ by macrophages, monocytes and astrocytes. Moreover, Tat can also stimulate the production of MIP 1 by astrocytes and increased levels of this chemokine can be found in the cerebro spinal fluid (CSF) of patients with HIV associated dementia (HAD). Similar findings have been shown in brain tissue of HIVE cases (Schmidtmayerova et al, 1996; Conant et al, 1998).

After viral entry to the cell the capsid is uncoated and proviral DNA is synthesized by the action of RT and some host components. This DNA, together with MA and Vpr, are incorporated into the host genome by a mechanism mediated by IN. The incorporated proviral DNA acts as viral RNA template. This RNA may be transported to the cell membrane and is incorporated into the progeny virion particle

or can serve as mRNA for Gag and Gag-Pol precursor proteins and/or for accessory proteins. Then these precursor proteins are proteolytically processed after assembling into the virion particle in the cell membrane (Reviewed in Ratner, 1996).

1.3 Diagnosis

The diagnosis of HIV-1 infection is based on virological and serological tests. Virological tests include the direct isolation of the virus from host tissues and identification of HIV RNA by PCR techniques, while the identification of HIV antibodies especially the core protein p24, by enzyme-linked immunoabsorbent assay (ELISA) or other methods such as western blot (WB) confirm the diagnosis. These methods are regarded as indirect methods.

Early diagnosis after exposure to HIV-1 is the major problem of ELISA, because this method is based on the detection of antibodies of IgG type, which may not be detected in serum for up to 6 weeks after infection. In children identification of antibodies of the IgA type are preferred for diagnostic purposes, due to transplacental transmission of IgG antibodies from a seropositive mother that can be identified in the serum of the infant for up to one year.

A number of so-called rapid serological tests for detection of HIV antibodies have been developed and they have been shown to have a high sensitivity and specificity ranging from 98.85% to 100% and 93.24% to 100% respectively (Phillips et al, 2000). Retrospective diagnosis of HIV-1 infection in tissue specimens can be made using polymerase chain reaction (PCR). This method is based on the amplification of HIV-1 DNA sequences of the HIV structural proteins and HIV enzymes, after DNA

extraction from formalin fixed paraffin embedded tissue. The specificity and sensitivity of this method is thought to be 100% and 95% respectively (Slavik et al, 1995).

1.4. Clinical Classification

Two different clinical classifications, defined separately by CDC and the World Health Organization (WHO) are currently in use (Appendix I). In the CDC classification a laboratory axis was introduced and the clinical groups were simplified into three categories instead of four as in the previous CDC classification (see CDC, 1986). The laboratory axis of this classification is entirely dependent on the CD4+ T cells numbers and allows the use of percentage of CD4+ T cells for classification purposes. In contrast the WHO laboratory categories include the total lymphocyte number.

Both grading systems are similar, although some minor differences can be identified (see Table 1.2). The importance of these grading systems is to allow comparisons between different cohorts of cases around the world. This has made possible the establishment of treatment and follow up protocols. Although most studies use AIDS as the end point of HIV disease, others studies are based on the type of AIDS defining event and some, even use the death of the patient as the end point, which makes comparisons difficult (Longini et al, 1993; Belanger et al, 1997; Morgan et al, 1997; Mocroft et al, 1997a; 1997b; Hendriks et al, 1998; Easterbrook et al, 2000).

Table1.2. Clinical classification: laboratory axis

Category 1	> 500 CD4+ cells/ml or 29%
Category 2	200-499 CD4+ cells/μl
Category 3	< 200 CD4+ cells/μl or 14%
WHO	
Stage A	>500 CD4+ cells x 10^6/L or >2000 total lymphoid cells
Stage B	200-500 CD4+ cells x 10^6/L or 1000-2000 total lymphoid cells
Stage C	< 200 CD4+ cells x 10^6/L or < 1000 total lymphoid cells

Source: CDC, 1992; Lifson et al, 1995.

1.5. Pathogenesis

The chronology of the progression of HIV infection is difficult to estimate due to the lack of accurate information about the date of infection. But it is accepted that the virus is recoverable from blood early after the infection. A documented case of an individual iatrogenically infected with HIV showed that the virus was recovered from the blood-stream by day 14 after inoculation and by day 15 the virus was cultured from a parietal lobe tissue sample (Davis et al, 1992).

In general terms, early HIV-1 infection is characterized by active viral replication, and transient but severe CD4+ T cell depletion, which is followed by the development of an acute immune response to HIV-1. The number of cytotoxic T lymphocytes (CTL) is the critical host factor limiting the extent of primary HIV-1 infection in both human and simian models of the disease (Daar et al, 1991; Clark et al, 1991; Lifson et al, 1991; Sheppard et al, 1993; Pantaleo et al, 1994; Reimann et al, 1994; Schmitz et al, 1999). Polyclonal activation of B lymphocytes, production of

neutralizing antibodies, binding of immune complexes to dendritic cells, production of cytokines and chemokines and activation of the natural killer cells (NK), are other host immune responses which efficiently lower the number of virions in peripheral blood. However, they fail to eradicate the infection (Klein et al, 1995; Pantaleo et al, 1995; Schacker et al, 1998). Clinical latency of HIV-1 infection follows and this can be sustained for years even in the absence of treatment. During this time, the host immune response deteriorates and depletion of helper T cells follows. Viral titres rise when T cells decline and viral replication in the lymph nodes (LN) correlates with increased systemic levels of cytokines such as tumour necrosis factor alpha (TNFα), granulocyte-macrophage colony stimulating factor (GM-CSF) and IL 6.

Current evidence about retroviral entry through mucosal surfaces, using other primate immunodeficiency virus such as SIV or simian/human immunodeficiency virus (SHIV) has demonstrated that the majority of infected cells express CD4 receptors and the appropriate HIV-1 co-receptor, usually CCR5 chemokine receptor. After the initial phase of HIV-1 infection a latency period characterized by a constant decline in CD4+ T cells and active, although slower, viral replication develops.

Viral replication usually takes place in LN and in other lymphoid tissue in the body such as gut associated lymphoid tissue (GALT) and spleen. This is explained by the trapping of HIV-1 virions in dendritic cells in those organs. This raises the possibility that certain co-receptors are expressed in greater quantities in some organs, or the local cytokine/chemokine production can attract infected lymphocytes to specific lymphoid organs (for a review see Salmi and Jalkanen, 1997; Berger et al, 1999; Haase, 1999). Macrophage derived chemokine (MDC), which is chemotactic for CD4+ cells and in particular for the helper 2 T lymphocytes (Th2) is expressed in

mucosal tissue. Secondary lymphoid tissue chemokine (SLC) is also a potent chemotactic for CD4+ T cells and is produced by secondary lymphoid tissue and thymus. It has also been shown that CD4+ T cells express some chemokine receptors such as CCR5 and CXCR4 in the case of memory T cells and CXCR4 in naïve T cells (Bonecchi et al, 1998).

An interesting fact was the discovery of change of co-receptor use by HIV, during the so-called latency period of the infection (Connor et al, 1997). During the primary infection, the HIV phenotype isolated from patients is the M-tropic (NSI, R5) virus. Shifts towards the more aggressive T cell tropic (SI, X4) virus in later stages of HIV disease is associated with CD4+ T cell depletion and a certain lack of sensitivity to chemokine inhibition of HIV infection. This change in HIV phenotype had been previously shown to occur (Schuitemaker et al, 1992), but a proper explanation for it was lacking at that time. Others suggested, that quantitative and qualitative changes in HIV were the most reasonable explanation for this phenomenon (Connor et al, 1993; Connor and Ho, 1994). Almost at the same time as the HIV co-receptors discovery, it was noted that chemokines effectively inhibited HIV infection in permissive cells (Oberlin et al, 1996; Bleul et al, 1996; Arenzana-Seisdedos et al, 1996; Choe et al, 1996; Cocchi et al, 1995). Further studies demonstrated that chemokine mediated entry is a separable function of the chemokine receptor (Farzan et al, 1997). The exact mechanism of HIV fusion and entry is complex; Berger and colleagues (1999) review it in detail. The chemokine receptor shift (Glushakova et al, 1998) from CCR5 to CXCR4 is one factor influencing the progression of HIV infection. It is known that this viral factor effectively influences the disease progression (Cheng–Mayer et al, 1988; Fenyo et al, 1988; Tersmette et al, 1988).

Other viral factors, probably some HIV variants with differential tropism for a specific cell type, could also be determinants for the progression of HIV infection (Patterson S. et al, 1998; Ball et al, 1994; Tanaka et al, 1997). Although changes in the hypervariable V1-V5 loops of gp120 have been implicated, some authors also suggest that these changes may be responses to certain host factors (Brew et al, 1996).

Among the host factors implicated in modifying HIV disease progression are:

1) Increasing age at time of infection (Belanger et al, 1997; Prins et al, 1999). This issue remains controversial, since age has been implicated as a major factor in HIV disease progression after the CD4+ T cells counts drop below 200 cells per ml. (Carre et al, 1998).

2) Gender. Women have a lower viral load than men, but their risk of progression to AIDS is higher (Farzadegan et al, 1998). This is still controversial because the association between gender and HIV viral load has not been found by other authors (Moore et al, 1999).

3) Route of transmission seems to be important since higher viral titers are found in blood than in semen. Different viral strains have also been identified in semen and in blood in men (Ball et al, 1999, Coombs et al, 1998) and in cervical secretions and blood in infected women (Poss et al, 1995). It is also important to note that CCR5 has been shown to be significantly increased in women undergoing progesterone treatment and in those with other sexually transmitted diseases apart from HIV infection (Patterson B.K. et al, 1998). Concurrent inflammation of the genital tract in men is associated with a significant increase of HIV provirus in semen (Xu et al, 1997). Intravenous inoculation of HIV has been investigated in blood transfusion

recipients in whom differences in progression to AIDS has been associated with the clinical stage of the donor (Ward et al, 1989), possibly reflecting a higher viral load in donors with lower CD4 counts. There are major difficulties in studying intravenous drug users due to lack of information about the time of infection and also due to the high incidence of death unrelated to AIDS. In this group of cases, there is discrepancy in defining the end point (AIDS or Death) among different cohorts of cases (Hendriks et al, 1998).

4) CD4 counts and viral load. These are major determinants of HIV progression to AIDS. This is particularly important when taken together with other demographic parameters such as age and risk factor. For example, the number of CD4+ cells at the time of sero-conversion was found to be lower in older intravenous drug users in the UK (Easterbrook et al, 2000). This may be explained by the physiological variation in CD4 counts and the inverse correlation between CD4 counts and viral loads. To make this phenomenon more confusing, it seems the CD4 depletion is unrelated to the viral replication as one would expect (Yu et al, 1994), but it seems that the viral replication is associated with immune cell activation (Theus et al, 1998). On the other hand, the plasma viral load is directly related to transmission of the infection (Fiore et al, 1997). Thus, before the availability of highly active anti-retroviral therapy (HAART), it was noted that treatment effectively reduced the HIV sexual transmission (Musicco et al, 1994). There is a complex association between the number of CD4+T cells, especially in the later stages of HIV-1 infection with opportunistic conditions, and these opportunistic conditions have been associated with a differential progression from AIDS to death (Mocroft et al, 1999; Morgan et al, 1997). Furthermore, prophylactic therapy against opportunistic conditions also

delays the progression to AIDS (Longini et al, 1993). It appears therefore, that there is a complex interaction between the host immune system and HIV viral load, which merits a more detailed discussion (see later).

5) The cytokine and chemokine milieu is also an important determinant of the progression of HIV infection. The chemokine effect on HIV-1 infection was previously mentioned. It has been shown that in some patients with high CD4 counts, there was an overproduction of the cytokines IL4 and IL10. These same patients had overproduction of interferon gamma (IFNγ) (Fakoya et al, 1997). More recently, it was demonstrated that IL 4 can up-regulate the expression of CXCR4 in CD4+ T lymphocytes, and that IL 10 has the opposite effect on CD4+ T lymphocytes. It is well known that IL 10 up-regulates the expression of CCR5 in human monocytes (Jinquan et al, 2000). The exact extent to which these and other possible cytokines influence the progression to AIDS remains to be clarified, but it seems as if the HIV virus itself modifies the response of mononuclear cells to cytokines.

6) Genetic factors are also important determinants of HIV progression. Soon after the description of HIV co-receptors, a 32 base pair deletion in an allele of chromosome 3, in the *CCR5* gene was identified. It was called 32 delta (32Δ). Homozygosity for this deletion was found in exposed uninfected (EU) individuals and hetrozygosity was found in a number of long-term HIV infected subjects (Dean et al, 1996). Similar results have been published by others (Bakshi et al, 1998; Quillent et al, 1998; Visco-Comandini et al, 1998). Other authors have shown that the protective effect of heterozygosity for the 32Δ was restricted to individuals infected by R5, NSI viruses, implicating that the shift of co-receptors by the HIV does not always occur (Michael et al, 1997). The discrepancy among results can be explained by the

number of cases studied, due to the low frequency of the 32Δ deletion. In the first report (Dean et al, 1996), the complete cohort of cases was 1955 and different groups of patients from several American cities were studied, while in the last study (Michael et al, 1997), a cohort of 406 cases was studied. After the discovery of the 32Δ on *CCR5*, single nucleotide polymorphisms on the *CCR2* gene (CCR2-64I) and in the stromal derived factor 1 (*SDF-1*), the major ligand for CXCR4, known as SDF-1-3'A, were identified (Smith et al, 1997; Winkler et al, 1998). These were shown to delay the onset of AIDS. This observation has been confirmed by others (Kostrikis et al, 1998). Single nucleotide polymorphism on the promoter region of *CCR5* gene (*CCR5* 59029 G/A), has a similar effect on HIV progression (for a review see Berger et al, 1999).

In summary, many factors directly or indirectly, isolated or in conjunction influence either the progression to AIDS or from AIDS to death.

1.6 T Lymphocytes

The overall state of immune deficiency is due to an imbalance between production and destruction of T cells. Direct cytotoxic effect of HIV-1 is not the most important factor accounting for the decline in CD4 T cells. Immune defensive mechanisms seem to play a decisive role in controlling the number of cells and the extent of HIV-1 infection.

1.6.1 T Lymphocyte Sub-populations

Surface markers can differentiate functional sub-populations of T cells. Naïve T cells express CD45RA and CD62L antigens, while memory T cells express CD45RO

antigens. Both naïve and memory CD4+ cells have been shown to be infected by HIV-1 (Woods et al, 1997; Ostrowski et al, 1999). During HIV-1 infection, CD45RO positive T cells are the preferred cells for viral infection. Memory T cells have shorter life spans than naïve T cells and proliferate in response to abundant antigen and/or as a result of IL 2 or IL 7 stimulation in an antigen independent manner. Furthermore, memory CD4+Tcells bearing the CD45RO+ receptor produce four to 19 times more infective competent virus than naïve CD4+T cells (CD45RA+) (Woods et al, 1997). This difference in cellular viral load was smaller in patients harboring X4 (SI) viruses indicating a compartmentalization of HIV in CD4+ T cells (Ostrowski et al, 1999).

Untreated HIV infection is associated with an increase in the number of memory/effector CD4+/CD8+ cells. In some HIV-1 positive individuals, however, an increased number of naïve CD45RA+ and CD62L+ cells is observed and this correlates with an increase in the size of the thymus on CT scan (McCune et al, 2000). Some lines of evidence suggest that in the setting of HIV-1 infection, CD4+ T cells are to some extent derived from stem cells. An alternative explanation for this finding is that the observed CD4+ T cells are derived from naïve CD45RA+ cells, which have a longer life span. This is supported by the presence of naïve T cells (both CD4+ and CD8+) in peripheral blood, several years after thymectomy of HIV sero-positive individuals (See Haynes et al, 2000 for a review).

CD4+ cells can be subdivided into T helper (Th) 1 and 2 lymphocytes. These lymphocytes produce different kinds of cytokines. Thus, Th1 cells produce IFγ, IL2, IL12, TNFα, which facilitate cell-mediated immunity. The subset of Th2 lymphocytes produce IL4, IL5, IL6, IL10, which are modulators of humoral immune

response. Cytokines of the Th1 type can also be secreted by CD8+ T lymphocytes but CD8+ T cells mediate their function mainly acting as cytotoxic cells. They are also known as cytotoxic T lymphocytes (CTL).

1.6.2 The Th1/Th2 Switch

It has been suggested that in the course of HIV-1 infection a switch from predominant Type 1 cytokine production, to a predominant antibody enhancing Type 2 cytokine production, is associated with loss of T helper function (Clerici et al, 1994), although, this has not been shown by others (Fakoya et al, 1997).

The type 1 cytokine IL 2 mRNA has been shown to be decreased in HIV sero-positive patients. This reduction of IL 2 mRNA parallels an increase in IFNγ mRNA (Fan et al, 1993). Type 2 cytokines have also been shown to be dysregulated in HIV positive cases. While no changes in IL 4 mRNA have been observed in HIV positive cases compared with HIV negative controls, an increase in IL 10 mRNA in HIV positive individuals has been observed even in the asymptomatic period (Fan et al, 1993; Fakoya et al, 1997). One of the mechanisms suggested for this cytokine dysregulation involves the loss of CD4+ T cells that is observed during HIV disease progression. This is based in the observation that CD8+ T cells have lower cytokine mRNA than CD4+ T cells in both HIV positive and negative individuals (Fan et al, 1993). Another proposed mechanism is that HIV infection impairs the ability of peripheral blood mononuclear cells to produce IL 2, IL 4, and IL 10 after stimulation (Fakoya et al, 1997).

1.7 HIV Dynamics

After primary HIV-1 infection, in untreated individuals, a rapid viraemia develops reaching its highest level at day 21 (Little et al, 1999). During this viraemia, HIV-1 is distributed all over the body especially the lymphoid tissue and brain (Davis et al, 1992; Haase, 1999). In animal models of the disease, the number of productively infected CD4 T cells significantly increases in lymph nodes and other lymphoid tissue by the second week after infection (Haase, 1999). The viral titres in serum of untreated individuals and in non-human primates double every 10 hours. It has been estimated by mathematical models that each infected cell can produce enough virus to infect 19 further cells (Little et al, 1999, Nowak et al, 1997). The half-life of virions in plasma has also been estimated to be 6 hours. After the infection of the cell, it takes 2.6 days for the infecting virion to produce infective virions in a second cell, as the life cycle of HIV-1 is 1.2 days (Perelson et al, 1996). Most of these results have been achieved by giving infected individuals protease inhibitors (PI), which can effectively block the infection of other cells. Using this approach, it has been estimated that the production of HIV-1 virions is around 10.3×10^9 each day (Perelson et al, 1996). These *in vivo* findings contrast with previous *in vitro* results (Clark et al, 1991), which suggested that 10^2 virions were produced every day with an estimated virus cycle of 3 to 4 days. Nonetheless, these *in vitro* studies have demonstrated a higher rate of HIV transmission through cell to cell contact than the rate of infection by cell free virus (Dimitrov et al, 1993). The half-life of infected cells has also been determined at approximately 2 days in humans and 1.4 days in non-human primates by using similar experimental designs (Wei et al, 1995; Nowak

et al, 1997). Interestingly, the number of cells to which an infected cell could potentially pass on infection in this animal model was significantly lower, ranging from three to five (Nowak et al, 1997).

The initial viraemia in untreated individuals declines around the fourth week after infection (Clark et al, 1991; Daar et al, 1991; Pantaleo et al, 1994). Similar findings have been described in Rhesus monkeys (Reimann et al, 1994). This spontaneous decrease in viraemia is associated with a strong CD8+ T cells response, which is thus regarded as a major factor in the initial control of the viraemia (Koup et al, 1994; Safrit et al, 1994; Borrow et al, 1994). Although the immune response was previously associated with the viral decline in plasma (Daar et al, 1991; Clark et al, 1991), a strong response of memory CTL specific to various HIV-1 antigens (Koenig et al, 1988; Mcelrath et al, 1994; Yang et al, 1996), was associated with a better prognosis and faster control of the initial viraemia (Safrit et al, 1994; Walker et al, 1987,1988; Borrow et al, 1994; Musey et al, 1997). Animal models demonstrated the strong association between CD8+ T cells and HIV viraemia. Rhesus monkeys infected with SIV showed a marked increase in viraemia when they were depleted of CD8+ T cells. An effective control of the viraemia was achieved by the reappearance of SIV specific CD8+ T cells (Schmitz et al, 1999). Early studies also associated CD8+ T cells with inhibition of HIV replication. These *in vitro* studies pointed towards soluble factors (Walker et al, 1986; Brinchmann et al, 1990), since the experimental model did not allow contact between the infected cells and the CD8+ T lymphocytes. In contrast, experimental models in which cell to cell contact was allowed revealed that the effect of CD8+ T cells on inhibiting HIV replication was more significant (Walker et al, 1991; Smith et al, 2000; Chun et al, 2001). In

similarly designed experiments if an excess of antibody against CC chemokine was added, the level of inhibition of viral replication due to soluble factors did not change substantially (Chun et al, 2001). Thus, the low viraemia observed in untreated HIV infected individuals is explained by the activation of the immune system, especially by a marked CD8+ T cells response, which apart from the emergence of various HIV antigen specific CTL, can produce a variety of chemokines which effectively block HIV infection in susceptible cells (Cocchi et al, 1995; Walker et al, 1986; Kannagi et al, 1990), as well as other soluble factors capable of suppressing HIV replication in monocytes and macrophages (Moriuchi et al, 1996). It has also been established that most of the HIV replication during this period takes place in lymphoid tissue (Pantaleo et al, 1993), where there is evidence that severe CTL reactions take place (Janossy et al, 1985; Cheynier et al, 1994).

In a number of patients an oligoclonal expansion of CD8+ CTL cells has been observed, which in some patients reach disproportionate levels similar to those observed in autoimmune reactions. These findings have been reproduced in animal models (Chen et al, 1995; Pantaleo et al, 1994).

1.7.1 Viral Reservoirs

During the primary viraemia, HIV is disseminated throughout the body, and in certain organs such as lymphoid tissue, and possibly brain, the virus survives for many years even in treated patients (Finzi et al, 1997; Chun et al, 1997b, 1998; Schrager and D'Souza, 1998; Zhang et al, 1999). These organs are regarded as sanctuaries for HIV. This phenomenon (which has been studied more thoroughly in lymphoid tissue) is due to the presence of latently infected cells harboring integrated

viral DNA. Thus, these cells are capable of producing infective virions upon activation. Furthermore, active HIV replication is suggested by the presence of un-integrated viral DNA in some resting CD4+ T cells (Biberfeld et al, 1986; Zack et al, 1990; Brinchmann et al, 1991; Bukrinsky et al, 1991; Michael et al, 1992; Pantaleo et al, 1993; Embretson et al, 1993; Cheynier et al, 1994; Chun et al, 1995; Chun et al, 1997b; Coombs et al, 1998; Ball et al, 1999; Hosmalin et al, 2001). Although HIV can enter quiescent T lymphocytes, the viral DNA production is inefficient (Zack et al, 1992). Most of these infected resting T lymphocytes accumulate in lymphoid tissue, probably as a result of the expression of cell surface receptors such as CD62L, which is regarded as a lymph node homing receptor. This mechanism also explains why infected CD4+ T cells disappear from circulation as well as the lymphadenopathy observed in some HIV positive individuals. Signaling through homing receptors can induce apoptosis. This adds another factor, which differs from the cytopathic effect of HIV, and accounts for the CD4+ T cell depletion observed during the course of HIV infection (Wang et al, 1999). Although the above mentioned mechanisms illustrate the dynamics of latently infected CD4+ Tcells in lymph nodes, the possible presence of latently infected cells in the brain, remains unexplained.

Follicular dendritic cells (FDC) play an important role in the progression of HIV infection by trapping HIV virions. Animal models have shown that the majority of infected cells are CD4+ T lymphocytes and these cells accumulate in the paracortical area of lymph nodes (Haase, 1999). HIV-1 DNA has been also identified in CD4+ T cells in the follicular mantle zone of the paracortical areas of lymph nodes. In contrast HIV-1 RNA was observed in the germinal centre in FDC (Haase, 1999;

Finkel et al, 1995). Lack of productive infection in FDC has been demonstrated in animal models (Haase, 1999). Thus, virions trapped in FDC perpetuate infection of CD4+ T cells. HAART controls viral replication in productively infected cells, but fails to eradicate HIV completely, especially because of latently infected CD4+ T cells. The level of virions detected in FDC in patients receiving HAART also declines (for review see Haase, 1999), but is not eliminated, suggesting that FDC are cellular reservoirs for HIV.

In summary, the existence of latently infected T cells is well-established in lymphoid tissue and has been suggested in brain. These latently infected cells may account for the active viral replication observed during all stages of HIV infection (Chun et al, 1997b). A reasonable explanation for the lack of conclusive evidence of latently infected cells in non-lymphoid tissue is the lack of relevant experimental models and the ethical difficulty in obtaining tissue for research from HIV infected patients.

With the advent of HAART, it has become possible to estimate the important role that latently infected cells play in the HIV-1 dynamics in the host. Following a rapid drop in viraemia during the first weeks of HAART, a second, slower, decline in viraemia is observed which is due to the presence of infected cells with a longer life span, and the activation of latently infected CD4+ T lymphocytes (Perelson et al, 1997).

The number of latently infected cells carrying replication competent virus is in the order of 10^7 cells (Chun et al, 1997b; Pantaleo et al, 1991), most of which are found in lymphoid tissue (Pantaleo et al, 1991).

The half-life of latently infected lymphocytes has been estimated by mathematical models to be between 10-20 days, or even as long as 80 days if the infected cells

carry a defective provirus (Nowak et al, 1997). The long life span of latently infected cells makes the eradication of HIV from infected patients very difficult.

Latently infected cells express very low levels of viral antigens, in this way escaping the immune surveillance (see below). Resting CD4+ T cells carrying replication competent virus, have been demonstrated in infected individuals treated with HAART soon after the initiation of symptoms (Chun et al, 1998). The life span of resting CD4+ memory cells can be as long as several years. The importance of these cells in the dynamics of HIV and the implication for viral eradication under combined anti-retroviral therapy has been discussed in recent reviews (Haase, 1999).

1.7.2. Viral Escape

Mutations in the HIV genome is another potential mechanism accounting for the high viraemia observed in the late stages of the disease. Thus viral mutations in the CTL recognition epitopes can protect viral variants from the CD8 mediated cytotoxicity. Selection pressures exerted by CTLs may be the cause for the emergence of more pathogenic HIV strains (Phillips et al, 1991; Pircher et al, 1990; Couillin et al, 1994, 1995; Borrow et al, 1997; Price et al, 1997) and SIV (Mortara et al, 1998), that can influence in a negative way the progression of the disease. The presence of a wide CTL response to various HIV epitopes may be indirect evidence of previous HIV successful escapes (Sewell et al, 2000; Moss et al, 1995; Sarah et al, 1998). Host factors that can influence the rate of viral escape include homozygosity for HLA class I alleles, which have been shown to be associated with more rapid disease progression. Conversely, the number of viral epitopes that can be recognized by CTL is greater when presented by heterozygous HLA molecules. Furthermore,

some HLA types directed against conserved viral epitopes such as gag and p24, are more likely to be found in long term non progressors or multi exposed uninfected individuals (Sarah et al, 1998; Harrer et al, 1996; Goulder et al, 1997; Brander et al, 1998). Immune escape has also been questioned by others (Ganeshan et al, 1997; Nietfield et al, 1995). These viral strategies fit with others previously described, such as latent infection in cellular reservoirs, down-regulation of MHC class I, concealment of the virus in immune-privileged sites and depletion of CD4+ T helper cells. But understanding the mechanisms of viral escape is of pivotal importance for the development of an effective vaccine. Thus, the ideal vaccine must be directed against several immunodominant regions of HIV proteins (gag, p24), which could elicit a strong CTL response against HIV, and at the same time render the viral escape more difficult (Sewel et al, 2000).

1.8 Mechanisms of CD4 depletion

The pre-symptomatic period of untreated individuals is characterized by low viral load, active viral replication, and sustained, although initially subtle, reduction in the CD4+ T cells.

Mechanisms for the CD4+ T cells decline during the course of HIV infection include the cytopathic effect of replicative competent virus, but as previously mentioned the number of productively infected cells is low, and the life span of these cells is short. Thus, the cytopathic effect of HIV replication cannot alone explain the profound decline of CD4+ T cells. Glycoproteins of HIV especially gp41 are necessary for

syncytium formation of infected CD4+ T cells and syncytium formation has been associated with apoptosis (Laurentcrawford et al, 1993, Ferri et al, 2000).

1.8.1. Apoptosis

Following the demonstration of apoptotic demise of HIV infected and uninfected cells (bystanders) (Finkel et al, 1995) and the induction of apoptosis by the HIV envelope and CD4 cross-linking (CD4XL) (Corbeil and Richman 1995; Hienkelein et al, 1995), the role of the Fas pathway was soon demonstrated (Katsikis et al, 1995) and confirmed by others (Kameoka et al, 1997).

1.8.1.1. Activation induced cell death (AICD)

Previous attempts to explain the CD4+ T cells decline showed that activation of infected CD4+ T cells was followed by demise of these cells (Zaguri et al, 1986). The type of cell death following activation of infected CD4+ T cells was characterized as apoptosis (Groux et al, 1992; Meyaard et al, 1992). Cross-linking of bound gp120 to CD4 receptor, followed by activation of the CD4+ T cells via T cell receptor (TCR), induces apoptosis of the infected cell (Banda et al, 1992; Oyaizu et al, 1993). This activation induced-cell death (AICD) was also observed in CD8+ T cells (Gougeon et al, 1993). HIV Env proteins such as gp 160 have been shown to induce up-regulation of Fas receptor on the lymphocyte surface and also to increase Fas mRNA on lymphocytes, upon CD4XL. Fas is a type I integral membrane protein which belongs to the TNF receptor superfamily and expresses an intracellular death domain. Fas ligand (FasL) is a type II transmembrane domain, which is homologous with TNF. Following FasL binding to Fas, apoptosis is initiated. Thus, it was demonstrated that AICD followed Fas activation (Oyaizu et al, 1994; Brunner et al,

1995; Alderson et al, 1995; Ju et al, 1995). Interestingly, CD4XL induced the production of certain Th1 cytokines such as IFNγ and TNFα only if the Th1 cytokine IL 2 and the Th2 cytokine IL 4 were not present in the culture media (Oyaizu et al, 1994).

Finally, AICD has been shown to occur after activation of T cells, since Th1 CD4+ T cells have been shown not to support HIV replication efficiently. It has therefore been proposed that this sub-population of CD4+ T cells is more vulnerable to AICD, leading to a preponderance of Th2 type cytokine productive CD4 and CD8 cells (Fakoya et al, 1997). It has also been shown that antibodies against IL 10 can block CD4 AICD *in vitro* (Estaquier et al, 1995). Apoptosis of CD4+ T cells was inhibited *in vitro* by adding antibodies against IL 10 and IL 4 to the culture media. Other Th 2 cytokines such as IL 6 have been shown to inhibit AICD (Ayroldi et al, 1998). Thus the role of Th2 cytokines in apoptosis seems to be selective. Nonetheless, an anti-apoptotic, "protective" effect of some Th1 cytokines such as IL 2 has been proposed (Estaquier et al, 1995). It is known that the CD4 molecule is a co-receptor for the T cell receptor (TCR) and CD3 complex (TCR/CD3). Stimulation of CD4 by this complex activates T cells and increases IL 2 production. CD4XL, before TCR stimulation up-regulates Fas, and triggers apoptosis. On the other hand, CD4+ T cells express Fas ligand (FasL) on their surfaces following CD3 stimulation. CD4XL was found to be associated with an increased susceptibility to apoptosis of CD8+ T cells suggesting the expression of Fas receptors on CD8+ cells. The induction of apoptosis through cell to cell contact is mediated by this mechanism. Effector cells (expressing FasL) can be CD4+ T cells, CD8+ T cells (Hanabuchi et al, 1994; Yang et al, 1997), or macrophages (Badley et al, 1996, 1997), which induce apoptosis by contact either

with other CD4+ T cells or CD8+ T cells expressing Fas receptor (Tateyama et al, 2000). HIV envelope glycoproteins cross-link with the CD4 receptor and, depending upon the maturation state of the cell, induce apoptosis and simultaneously abrogate Th1 functions. Th1 cytokine production also enhances the secretion of the FasL molecule (sFasL), which is normally preformed and released upon activation of T cells (Tanaka et al, 1995; Martinez-Lorenzo et al, 1996). Plasma levels of sFasL correlate negatively with HIV disease progression. Thus, the decline of sFasL directly correlates with T cell depletion and specifically with Th1 functioning T cells (Bahr et al, 1997).

Further studies on Th1 cytokines and apoptosis demonstrate a differential susceptibility to apoptosis among the Th1 producing T cells. AICD rate was higher among TNFα producing cells than for IFNγ producing cells and the cells less susceptible to apoptosis were those producing IL2. In HIV infected individuals there is a diminished representation of Th1 cytokine producing T cells. A reduction in IL 2 and TNFα producing T cells has been demonstrated and, even more important, the disappearance of IL2 producing cells correlates with HIV disease progression and depletion of naïve CD4 cells. (CD4+ CD45RA+) (Ledru et al, 1998). In summary, an HIV direct cytopathic effect in CD4+ cells accounts for some of the CD4 T cell depletion observed in AIDS, but this alone is insufficient to cause all of the T cell loss. Apoptosis of both CD4+ and CD8+ T cells seems to be more important. Two types of apoptotic demise can be observed in CD4+ and CD8+ T cells. One, AICD, is activation dependent triggered by TCR/CD3 CD4XL and the second is also Fas mediated requiring contact between cells. In the last setting, effector cells can be CD4+ or CD8+ T cells or macrophages. Fas expressing cells, HIV infected or

bystander cells can be susceptible to apoptosis induced upon contact (Badley et al, 1996, 1997).

1.8.1.2. TNFα receptor II

Another apoptosis pathway has been also observed in CD8+ T cells. These CD8 cells express TNFα receptor II (TNFαRII) on their surface. Infected macrophages express membrane bound TNFα (mbTNFα) as well as FasL. By contact with its receptor mbTNFα induces apoptosis of CD8+ T cells. Cross-linking between gp120 and the CD4 receptor or SDF-1 (stromal derived factor 1) cross-linking with the HIV co-receptor CXCR4 is sufficient stimulus to up-regulate mbTNFα (Herbein et al, 1998). This mechanism might be more relevant in preventing the infection of new cells in later stages of HIV infection. In that setting the phenotypic switch of HIV from predominantly R5 viruses in primary infection to the more pathogenic X4 viruses has occurred.

1.8.1.3. Caspase pathway

The caspase pathway of apoptosis is a well-studied phenomenon in lymphoid cells and has also been linked to the mechanism of T cell depletion observed in the course of HIV-1 infection. For example, apoptosis induced by cellular contact of infected and bystander cells was found to be blocked by interleukin 1β converting enzyme (ICE) inhibitors, but this compound did not abrogate the syncytium formation, although both events share the same pathogenic mechanism (Ohnimus et al, 1997). ICE was shown to be similar to the CED-3 protein of the C elegans, which is encoded by the *CED*-3 gene also known as the death gene (Yuan et al, 1993). ICE was the first caspase identified; thus it was renamed caspase 1. Almost

simultaneously, the Fas and FasL (also known as CD95) pathway was linked with the caspases pathway. It was shown that ICE inhibitors also blocked Fas mediated apoptosis of CD4+ and CD8+ T cells in asymptomatic HIV-1 infected individuals, as well as AICD in some but not all HIV infected individuals (Katsikis et al, 1997). Subsequent reports linked T cell apoptosis to different caspases such as caspase 3 (Liegler et al, 1998) and caspase 3 and 6 (Cicala et al, 2000). Interestingly, low caspase 3 activity was found in HIV infected individuals who progress slowly towards AIDS. Apoptosis of lymphoid cells in the same individuals was shown to increase when mitogens were added to the culture media suggesting a less severe depletion of lymphocytes and a link between apoptosis and cell cycle respectively (Liegler et al, 1998).

The apoptotic process is well explained in an increasing number of reviews as the number of mechanisms and molecules involved are identified (Nicholson and Thornberry, 1997; Kidd, 1998; White, 1996; Teodoro and Branton, 1997; Barry and McFadden, 1998; Vaux and Strasser, 1996; Budihardjo et al, 1999). A brief description of the caspase pathway is as follows: Caspases are cysteine proteases, which share a specific cleavage site after aspartic acid residues. The name caspase comes from C for cysteine, and aspase from aspartic acid. More than 14 caspases have been identified. They can be divided into three main groups: those involved in inflammatory responses such as caspase 1 and 11 (ICE like), those involved in the initiation of the apoptotic process such as caspase 8, and those effector caspases such as caspase 3, 6, 7 and 9. Two main pathways of caspase activation are also well established, the intrinsic and extrinsic pathways. The extrinsic pathways trigger apoptosis after the binding of a ligand to its receptor. These ligands belong to the

TNF family proteins such as FasL (CD95L). Receptors also belong to the TNF/nerve growth factor (NGF) receptor super family. These death receptors include Fas (CD95/APO1).

The intrinsic pathway originates in the mitochondria, which release cytochrome C, which in presence of ATP can activate caspase 3. Cytochrome C is released by the mitochondria by a variety of stimuli such as DNA damaging agents or death receptor stimulation. Cytochrome C needs to interact with pro-caspase 9, to activate caspase 3. The most important step in the intrinsic pathway of caspase activation is the release of cytochrome C from the inter-membrane space of mitochondria. The Bcl2 protein family plays a key role in modulating the release of cytochrome C from mitochondria.

Going back to the extrinsic pathway, the events following the binding of the ligand with the appropriate receptor are governed by a sequential activation and recruitment of a series of intracellular receptor-associated proteins that facilitate the caspase activation. The Fas pathway exemplifies this. After binding with its ligand, the intracellular region of Fas that contains a death domain (DD) recruits the Fas-associated protein with death domain (FADD). Thus, interaction of both DD, one from Fas and the other from FADD, form the death-inducing signaling complex (DISC). The other end of FADD (N terminal fragment) is known as the death effector domain (DED), which interacts with the DED domains (also in the N terminal fragment) of caspase 8 or 10. These are then proteolytically processed to the active form. Activated caspase 8 or 10 in turn activates other caspases downstream, such as caspase 3, which in turn activates nuclear proteins (caspase 3 cleaves nuclear mitotic apparatus protein and induces shrinking and fragmentation of the nuclei).

Recently, two types of cells with different rates of response to the caspase pathway have been reported and are currently accepted. Type I cells respond to Fas/CD95 (extrinsic pathway) without the involvement of mitochondria (intrinsic pathway), while Type II cells respond to both extrinsic and intrinsic pathways.

There are still conflicting reports about the response to one or other death receptors among activated T lymphocytes. The increasing number of proteins involved in triggering apoptosis or transducing the death signal to caspases, or modulating the apoptotic process will explain the discrepancies observed today. For example, the Fas pathway has been implicated in AICD of CD4+ and CD8+ T cells but the spontaneous apoptosis of peripheral blood cells of HIV infected individuals, has been shown to be Fas independent (Kaplan and Sieg, 1998). This suggests that the intrinsic pathway may also be involved in HIV associated apoptosis (Ferri et al, 2000; Rasola et al, 2001; Conti et al, 1998). It appears that different pathways may be involved in the lymphoid depletion and this depends upon the maturation state of the lymphoid cell committed to apoptosis (for review of discrepancies about apoptosis in HIV-1 infection see Kaplan and Sieg, 1998). It is interesting that blocking both caspase pathways in PBMCs and the anti-apoptotic actions of some HIV proteins enhances HIV replication (Chinnaiyan et al, 1997).

1.8.1.4. CTL mediated apoptosis

Other apoptotic pathways have been described in relation to the action of CTL against HIV infected and bystander T cells (Kagi et al, 1994; Kojima et al, 1994; Lowin et al, 1994). One of them involves a sequential release of CTL cytoplasmic granules after CTL recognition of antigen bearing cells. The other is mediated by

FasL expression on CTL cells as mentioned earlier. HIV infected CD4+ T cells express Fas on their surfaces by action of some HIV proteins such as gp 120 and Tat. In the latter pathway, the bystander cell lysis seems to be mediated by infected CD4+ cells, with little if any help of antigen specific CTL (Shankar et al, 1999). CTL exocytic degranulation can also lyse antigen-bearing cells by the action of perforin (Su et al, 1994) which forms a complex that attaches to the cellular membrane and opens pores of approximately 16nm. This mechanism alone can cause osmotic lysis of the cells, but even more interesting is the association of perforin with a serine protease known as granzyme B (Chinnaiyan et al, 1996). This serine protease cleaves substrates after an aspartic acid (D) residue. This cleavage specificity is shared with other proteases such as caspases (see above). Granzyme B is also contained in CTL cytoplasmatic granules and is necessary for the fragmentation of nuclear DNA. It is transported to the cellular nucleus where, by unknown mechanisms, it accumulates. Granzyme B also cleaves and activates caspase 3 and 9, which are the effector caspases (Jans et al, 1996; Heusel et al, 1994; Darmon et al, 1994; Su et al, 1994; Shresta et al, 1997).

In summary, apoptosis is one mechanism by which T cells are depleted during the course of HIV-1 infection. Evidence for caspase activation by both pathways has emerged and these findings that appear controversial, can be explained by the cytokine production by lymphocytes, although this remains speculative. Another possibility is that the cytokine milieu of an individual patient could modulate the response to one caspase activation pathway or the other. Evidence for this comes from the up-regulation of FasL in CD8+ T cells by RANTES (Hadida et al, 1999).

The other possibility is the existence of a different caspase activation pathway that has not yet been completely characterized. Evidence for this comes from the demonstration of two different apoptosis pathways in T cells, triggered by tumour cells. In this *in vitro* model, T cells were shown to be differentially susceptible to a panel of inhibitors of the mitochondrial pathway, even if agonistic Fas antibodies were used (Gastman et al, 2000). The fact that some anti-neoplastic drugs can activate caspase 8 by a mechanism different from the Fas receptor also supports this theory (Wieder et al, 2001). In any case the net result of HIV disease progression is a profound decline in both CD4+ and CD8+ T cells.

1.8.2. T lymphocyte production

It is recognized that thymopoiesis is present throughout life and it has been linked to the immune reconstitution observed following HAART (McCune et al, 2000). It has also been shown that the thymic involution rate is greater up to 40 years of age, but after that, the involution rate declines (Haase, 1999). Functional thymic tissue has been observed in HIV-1 infected patients (Haase, 1999), supporting the concept that naïve T cell (both CD4+ and CD8+) production is conserved for life and during HIV-1 infection, although alteration in bone marrow CD34+ T cell progenitors has also been found (Haase, 1999).

It is clear that constant production of T cells occurs alongside T cell apoptosis throughout HIV infection. When the balance tilts towards the latter, T cell depletion ensues. Thus during the pre-symptomatic period, a balance between production and destruction of T cells is the rule, but when that balance is disrupted, an increase in viraemia is observed. There are several mechanisms that explain this. As mentioned

before, the CD8+ T cells response is associated with the control of primary viraemia (Borrow et al, 1994; Koup et al, 1994; Safrit et al, 1994; Walker et al, 1987; Walker et al, 1991; Pantaleo et al, 1994), and throughout the disease in humans (Hosmalin et al, 2001; Smith et al, 2000; Chun et al, 2001) and non-human primates (Matano et al, 1998). Consequently, impaired function of circulating CD8+ T cells can account at least partially for the increase in viraemia observed in the end stage of HIV-1 infection. It has been shown that CD8+ T cells in chronic HIV infected individuals are functionally impaired (Shankar et al, 2000). Destruction of CD4+ helper cells is an indirect mechanism for impairing CD8+ T cells function.

1.9 HIV and CNS

Studies of lentiviral infection in both human and various animals have shown that all these viruses may invade the CNS causing pathological lesions. As other lentiviruses, HIV can cause neurological disorders, which have been grouped into those of the peripheral nervous system, and disorders of CNS. By the time of seroconversion, some patients (up to 10%) develop acute aseptic meningitis, characterized by headache, stiff neck, fever and pleocytosis. Anti HIV antibodies in the CSF are usually present (reviewed in Janssen et al, 1989).

In the CNS, HIV can cause a number of neurological, neuropsychological and psychiatric illnesses all grouped under the general term of HIV associated cognitive/motor complex (Janssen et al, 1991). According to its severity, this HIV associated cognitive/motor complex, can be subdivided into HIV associated

dementia complex and HIV associated myelopathy as the most severe forms and HIV associated minor cognitive/motor disorder for less severe manifestations (Budka et al, 1991). Diagnostic criteria for these conditions have been defined by the American Academy of Neurology AIDS Task Force (AANATF) (Janssen et al, 1991). Among the clinical features of the HIV associated cognitive/motor complex, cognitive impairment is characterized by forgetfulness, difficulty with concentration, speech difficulties, mental slowness, disorientation, apathy, lethargy, loss of sexual drive and alterations in the response to emotions. The motor component of this complex is characterized by weakness of legs, unsteady gait and tremor (Janssen, 1991).

Some of the peripheral nerve disorders can be observed in patients in the pre-symptomatic stage, while the CNS manifestations occur when the immunodeficiency develops. Other neurological disorders related to opportunistic conditions, both neoplastic and infectious in nature can overlap or dominate the neurological or neuropsychological clinical manifestations of AIDS patients. In more advanced disease (AIDS), neurological complications develop in up to a third of the adults and half of the children (Janssen, 1989).

It is accepted that entry of HIV into the CNS may occur through the passage of infected lymphocytes/monocytes across the blood brain barrier (BBB), although this has not been conclusively demonstrated. HIV enters the CNS early in the course of the infection, as it was demonstrated in one study in which HIV-1 was isolated from the brain 15 days after iatrogenic HIV-1 infection (Davis et al, 1992).

In the CNS microglial cells and macrophages are primarily infected (Koenig et al, 1986; Wiley et al, 1986; Gabuzda et al, 1986). Some of these macrophages fuse and

form multinucleated giant cells (Budka, 1986; Wiley et al, 1986) which are a histological characteristic of advanced (late stage) HIV disease (Budka et al, 1991). Several factors have been studied in relation to the development of neurological and neuropsychological abnormalities in the course of HIV infection. These factors can be divided into host factors, in particular, the integrity of the BBB, the host immune status and the type of infected cells in the CNS. Viral factors include the presence of viral RNA in the brain, the presence of viral proteins in the brain (Tat, gp120, and Nef and Ref) and viral variants found in the CNS. Indirect mechanisms have also been implicated in the development of neurological abnormalities, mostly related to the inflammatory process and chemical mediators and excitatory neurotransmitters such as quinolinic acid in the brain which can cause damage to the white matter, oligodendrocytes, astrocytes and neurons. Other factors that have also been found to be related to the development of neurological complications in HIV infected individuals include anti-retroviral therapies as modifiers of the HIV evolution.

HIV associated dementia (HAD), occurs after the establishment of immunodeficiency, is associated with short survival, and usually presents late in HIV disease (McArthur et al, 1993, Karlsen et al, 1995; Price et al, 1999; Ellis et al, 1997b). It has been shown that patients receiving nucleoside reverse transcriptase inhibitors (NRTIs) have a temporary improvement on neuropsychological tests, lasting for 6 months, probably reflecting the transient HIV suppression achieved by NRTIs. Furthermore, a significant correlation between neuropsychological performance and CD8+ T and CD4+ T cells counts has been shown using logistic regression analysis.

Other studies have shown low CD4 counts and high p24 antigens in the CSF and plasma in demented patients (Royal et al, 1994; Singer et al, 1994; Heaton et al, 1995; Ellis et al, 1997a; Dal Pan et al, 1998; Stankoff et al, 1999). In children, cortical atrophy on computed tomography correlated with HIV viral load in CSF (Brouwers et al, 1995, 2000) and dementia was found less likely in patients with at least 260 CD4+ T cell/μl blood (Cornelisse et al, 2000). All these observations indicate that immunodeficiency is associated with the development of cognitive impairment and that HIV disease progression is accompanied by HIV viral replication in the CNS.

A degree of cognitive impairment has also been shown in HIV positive asymptomatic individuals (Lunn et al, 1991; Bornstein et al, 1992, 1994; White et al, 1995) and abnormalities on MRI have also been described in HIV positive asymptomatic cases (Rosci et al, 1992) although the contrary has also been reported (McAllister et al, 1992). Even HIV RNA has also been found in the CSF of HIV positive pre-symptomatic cases (Ellis et al, 2000). These findings are difficult to explain but they may suggest that neurological and neuropsychological abnormalities develop early in the course of HIV disease, although caution in the interpretation of these findings must be exerted due to the methodological differences between different research groups and because a clear explanation of the pathogenesis of HAD is still lacking. Other researchers however, have suggested independent dynamics between viral strains derived from systemic sources and those derived from CNS. This is supported by abnormalities of the BBB that have been implicated in the passage of HIV infected cells into the CNS. It has been suggested that the initial passage of infected cells through the BBB is due to the increased expression of

adhesion molecules on activated HIV infected macrophages and endothelial cells. *In vitro* studies have shown that activated monocytes induce expression of the adhesion molecule E-selectin on co-cultured endothelial cells by cell to cell contact. Activated infected monocytes also express E-selectin and vascular cell adhesion molecule-1 (VCAM-1). A soluble factor from infected monocytes was suggested to induce expression of adhesion molecules in endothelial cells in transwell assays (Nottet et al, 1996). This *in vitro* evidence may explain the passage of infected monocytes into the brain across the BBB.

The persistent migration of inflammatory cells into the brain has been suggested as an important pathogenic mechanism for developing HAD. Several lines of evidence support the constant recruitment of inflammatory cells into the brain.

In HIVE cases, beta chemokines such as monocyte chemotatic protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α) and regulated on activation normal T cell express and secreted protein (RANTES) have been identified in brain macrophages. Production of IL8 and 10KDa INFγ inflammatory protein 10 (IP-10), which is chemotactic for inflammatory cells have been shown in astrocytes by immunohistochemistry and double label immunofluorescent laser confocal microscopy (Sanders et al, 1998) and it was suggested that they were involved in the recruitment of inflammatory cells. Disruption of the tight junctions of the blood brain barrier has also been demonstrated in HIVE (Dallasta et al, 1999) and in HAD cases (Boven et al, 2000), and this correlated with monocyte infiltration in HAD patients. Activated microglial cells have been shown to increase migration of monocytes through artificial BBB and in patients affected by HIVE. Microglial activation correlated with HIV infection, chemokine expression and transendothelial migration

of monocytes (Persidsky et al, 1999). In the CSF, activation of matrix metaloproteinase 9 (MMP-9), a proteinase capable of degrading components of the cellular matrix of the BBB, was found significantly correlated with CSF cell counts, suggesting a role of this proteinase in the transendothelial migration of inflammatory cells into the brain. The importance of these findings is that they support the current hypothesis that in advanced HIV infection, systemic inflammatory cells can migrate into the brain and exacerbate the inflammatory milieu in the CNS, leading to cognitive impairment.

1.9.1. CNS Viral Reservoirs

The role of different cell types of the brain during the course of HIV infection is a host factor of major importance in the development and progression of HIV associated cognitive/motor complex. One unresolved issue in this context is the presence of HIV in the brain. One view is that a transient infection occurs, with active viral replication in the early infection followed by clearance of the virus in the subsequent stages of HIV disease, and reappearance in AIDS. This view is supported by findings on simian immunodeficiency models in which SIV has been demonstrated in perivascular macrophages, which sustain productive infection in early stages of SIV infection, but thereafter, active viral replication is not found until immunodeficiency develops (Williams et al, 2001). It has been suggested that during late stages of HIV infection, a higher number of activated infected monocytes are in the circulation and they can favor the expression of adhesion molecules in endothelial cells of brain microvasculature. In that way, infected monocytes gain entry into the brain in late stages of HIV disease (Gartner and McArthur, 2000). This

hypothesis argues against previous evidence showing that not only macrophages, but microglial cells also, are productively infected (Takahashi et al, 1996; Stanley et al, 1994).

1.9.1.1. Microglial Cells

Microglial cells have long life spans and it is believed that they act as reservoirs for HIV. Several lines of evidence suggest that HIV replication is maintained during the asymptomatic period as shown by the presence of HIV RNA in the CSF of HIV positive cases in early HIV disease (Ellis et al, 2000). HIV-1 pro-viral DNA has been observed in the brain of asymptomatic cases (Gray et al, 1996). *In vitro* models have also shown that viruses isolated from acutely infected patients can infect both microglial cells and macrophages, but some of the viruses isolated showed preference for one of the other type of cells. After several passages, one isolate was further adapted to microglial cells inducing vacuolization and syncytium formation in cultured microglial cells. This isolate also showed variations on the amino acid sequences of the hypervariable region V3 of the gp120 similar to those observed in isolates from demented patients suggesting that viruses evolve in the CNS after early infection, resulting in microglial tropic viruses (Strizki et al, 1996; Smit et al, 2001). Another *in vitro* study has shown that HIV can replicate in microglial cells for up to 70 days post infection. This supports the concept of viral reservoirs (Ioannidis et al, 1995). Nevertheless, uncertainty still exists about the presence and source of the virus in the CNS during the asymptomatic stages of HIV infection.

1.9.1.2. Astrocytes

It was suggested that other cell types within the CNS can act as reservoirs of HIV. Astrocytes have been claimed to be productively infected *in vivo* (Ranki et al, 1995) but subsequent *in vivo* studies have failed to reproduce this finding (Takahashi et al, 1996; Thompson et al, 2001). *In vitro* studies have shown that HIV-1 infection of astrocytes is transient and rapidly becomes non-productive (Gorry et al, 1998; Fiala et al, 1996; Brengel-Pesce et al, 1997). Nevertheless, the presence of HIV regulatory proteins in astrocytes *in vivo*, is widely accepted and it is considered to be important for the development of HAD (Tornatore et al, 1994; Thompson et al, 2001). It has been hypothesized that this restricted infection of astrocytes may manifest clinically due to dysfunction of important metabolic actions of astrocytes such as glutamate buffering of perineuronal microenvironment. Alterations in the transport of excitatory amino acids such as glutamate and aspartate have been shown in cultured astrocytes infected with HIV-1. This alteration was shown to be transient, since the carboxyl-terminus fragment of gp41 was shown to mediate this dysfunction, but this transmembrane glycoprotein is expressed transiently in cultured astrocytes (Kort, 1998). The role of astrocytes as reservoir for HIV is still an open question, since although most *in vitro* and *in vivo* studies have demonstrated early regulatory proteins and gene expression, late gene (env-gag) activation *in vivo* in non-human primate models has been unsuccessful (Guillemin et al, 2000).

1.9.1.3. Neurons

Most studies have failed to detect HIV-1 neuronal infection *in vivo*, and it is generally considered that neurons are not susceptible to HIV infection (Takahashi et

al, 1996). Thus, the cognitive impairment observed during the course of HIV infection is probably mediated by indirect mechanisms of neuronal loss. Neuronal loss and reduced synaptic density and HIV viral load have been shown to correlate with cognitive impairment (Everall et al, 1999).

1.10 Mechanisms of neuronal loss

1.10.1. Apoptosis

One of the suggested mechanisms leading to neuronal loss is apoptosis. Early studies using rat cortical cell cultures pre-incubated with gp120 demonstrated DNA fragmentation at internucleosomal linkers characteristic of apoptosis. This change was prevented by N-methyl-D-aspartate receptor channel antagonists (Muller et al, 1992). Another viral protein, Tat has been shown to trigger neuronal apoptosis. Several mechanisms for Tat-induced apoptosis have been suggested and include the activation of prostate apoptosis response 4 (PAR-4), caspase activation and oxidative stress (Kruman et al, 1998, 1999). The HIV-1 accessory protein Vpr has also been shown to induce apoptosis of rat and human neuronal cell cultures, possibly by activation of caspase 8. This suggests the involvement of the extrinsic pathway of caspase activation. Other *in vitro* studies have shown that apoptotic cell death including not only neurons but astrocytes and endothelial cells is a late event, occurring 1-2 weeks after the viral production peaked suggesting an indirect mechanism possibly mediated by soluble factors (Shi et al, 1996).

Among the soluble factors which induce apoptosis of cultured neuronal cell lines are platelet activating factor (PAF) and TNFα (Talley et al, 1995; Pulliam et al, 1998;

Perry et al, 1998). It has been shown that TNFα-induced neuronal apoptosis can be partially prevented by Bcl2 protein, suggesting that the intrinsic (mitochondrial) pathway of caspase activation is involved (Pulliam et al, 1998). This can be explained by an indirect effect of TNFα possibly involving oxidative stress and/or glutamate toxicity. Activated or infected macrophages and microglia secrete TNFα, which may impair astrocyte uptake of exitotoxins such as glutamate. It is accepted that TNFα inhibits glutamate uptake by astrocytes leading to an accumulation of this substance in synapses (Fine et al, 1996). Over activation of glutamate receptors can activate caspases and generate reactive oxygen species and lipid peroxidation, which can also induce apoptosis (Tenneti et al, 1998). Another viral protein gp120 has been shown to activate Na+/H+ exchange in astrocytes to release glutamate and potassium *in vitro* (Benos et al, 1994; Holden et al, 1999; Patton et al, 2000). This is followed by increased intracellular concentration of Ca+, which can stimulate the release of cytochrome C from mitochondria and subsequent caspase activation. TNFα potentiates the pro-apoptotic effects of Tat, and this was partially inhibited by antioxidants (Shi et al, 1998). Recent *in vitro* studies have corroborated the neurotoxic effects of glutamate (Jiang et al, 2001), but *in vivo* studies, however, have shown no correlation between CSF levels of glutamate with HIV-1 infection of HAD (Espey et al, 1999).

In vivo studies using brain sections from human and non-human primates have shown the presence of apoptotic neurons and astrocytes (Gelbard et al, 1995; Petito and Roberts, 1995; Adamson et al, 1996; An et al, 1996; Adle-Biassette et al, 1995, 1997, 1999; Thompson et al, 2001) in various areas of the brain including frontal lobe, basal ganglia, temporal hippocampus, thalamus, midbrain and cerebellum.

Apoptosis was assessed by a combination of morphological and different techniques, which recognize internucleosomal DNA fragmentation either, by gel electrophoresis of DNA, (Adle-Biassette et al, 1995) or in situ end labeling. Both of these methods have been shown to be non-specific for apoptosis, since DNA fragmentation may also be detected in necrosis (Grasl-Kraupp et al, 1995; Sastry and Rao, 2000). Furthermore, glutamate receptor over-stimulation is one of the mechanisms suggested for apoptosis induction, but this can also induce necrosis of neuronal cell cultures (Ankarcrona et al, 1995). This can explain apparently contradictory reports about the topographical localization of apoptotic cells *in vivo* such as perivascular localization (Petito and Roberts, 1995), or not related to areas of vascular vulnerability (An et al, 1996). In SIV infected macaques, cells with DNA fragmentation were seen associated with perivascular inflammatory infiltrates (Adamson et al, 1996). Similar findings have been reported in man (Gelbard et al, 1995). Other groups did not find such associations but apoptotic neurons were seen more frequently in the vicinity of multinucleated giant cells (Adle-Biassette et al, 1999). Recently, evidence of DNA damage and repair in cases with HIVE was shown not to be associated with microglial nodules or HIV infected macrophages (Wiley et al, 2000), suggesting either the involvement of soluble factors in this process or the presence of a distinct apoptosis-inducing signal. It has been demonstrated *in vitro*, that the apoptotic mechanisms can be activated in synapses and dendrites, which may explain the synapses loss and neuronal death observed in this and other neurodegenerative processes (Mattson et al, 1998; Mattson, 2000).

In summary, neuronal apoptosis can be triggered by the intrinsic and extrinsic pathways of caspase activation in the setting of HIV infection. The signals involved

in these processes can be exerted in the vicinity of apoptotic cells or produced distantly in synapses and dendrites. The inflammatory process (and its mediators) associated with HIV infection, at least in the late stages of the disease as well as some viral proteins, or more probably the two factors acting together, generate the initial stimulus for inducing apoptosis. The excitotoxicity caused by over-stimulation of ionotropic glutamate receptors and especially the NMDA, can damage DNA and result in apoptosis or necrosis with consequent neuronal and glial cell loss.

1.10.2. Cytokines and Chemokines

Another host factor, which is relevant in the establishment of cognitive impairment, is the inflammatory reaction in the CNS. The levels of a number of cytokines and chemokines and other chemical mediators of inflammation have been found to be significantly elevated in the CSF of cases with HAD. Furthermore, differential expression of chemokine receptors in different areas of the brain and different types of cells has been shown *in vivo*.

Early during SIV infection of macaques, mRNA expression of IL1β, TNFα and IL6 in the CNS have been reported. This pattern of cytokine gene expression was shown to be coincident with reactive gliosis and the development of myelin lesions. It was also shown that these morphological changes were not associated with the number of productively SIV infected cells or the number of T cells infiltrating the brain (Boche et al, 1999). In this animal model early infection of perivascular macrophages occurs (Williams et al, 2001), suggesting that the source of these cytokines are macrophages. In other animals, HIV-1 infected macrophages were inoculated into the basal ganglia of severe combined immunodeficiency mouse model. Inhibitors of

platelet activating factor and matrix metaloproteinase inhibitors (which also inhibit TNFα release) were also administered in combination to the mice. This resulted in a marked reduction of reactive gliosis and microglial activation independently from viral replication demonstrating that the inflammatory mediators are associated with the morphological changes observed in late stages of HIV infection (Persidsky et al, 2001).

Different inflammatory mediators including cytokines, chemokines, metabolites of arachidonic acid (prostaglandins, PAF), nitric oxide, reactive oxygen species, among others, have been shown to be secreted or their genes expressed *in vitro* and in the brain of HIV positive cases in late stages of HIV infection (Wesselingh et al, 1993, 1997; Achim et al, 1993; Griffin et al, 1994; Seilhean et al, 1997; Yeung et al, 1995; Wilt et al, 1995; Vitkovic et al, 1995; Nath et al, 1999). The sources of these inflammatory mediators are macrophages, microglial cells and astrocytes.

Reactive oxygen species have been found in the brain of HIV infected cases. Superoxide dismutase was found in 70% of HIV p24 positive macrophages and inducible nitric oxide synthase RNA expression in astrocytes was found significantly increased in HAD cases, suggesting the production of superoxide in the CNS of these cases (Boven et al, 1999). In macaques, increased expression of inducible nitric oxide synthase has been shown to correlate with dendritic injury (Li et al, 1999). Chemokines and their receptors have also been shown in brain tissue of HIV infected individuals. It is believed that these chemokines are responsible for the recruitment of monocytes, macrophages and T cells. CSF levels of monocyte chemotactic protein 1 (MCP-1), RANTES, and macrophage inflammatory protein 1 alpha and 1 beta (MIP-1α-MIP1β) have been shown to be higher in cases with HAD than in HIV

positive individuals without neurological complications (Kelder et al, 1998, Letendre et al, 1999). Furthermore, MCP-1 correlated with HIV viral loads in CSF and the severity of dementia (Kelder et al, 1998). Immunohistochemical studies have also demonstrated the presence of MCP-1, MIP-1α, and RANTES in brain macrophages, which correlated with HIVE (Sanders et al, 1998). Similar results have been obtained in macaques infected with SIV (Sasseville et al, 1996; Westmoreland et al, 1998). The chemokine receptor CCR5 was immunohistochemically demonstrated in macrophages and microglial cells, while CXCR4, immunostaining was observed in macrophages, microglia, neurons and astrocytes (Sanders et al, 1998).

Other chemokine receptors such as CCR3 together with CCR5 (which are expressed by microglia), are believed to play a major role for HIV infection of these cells (He et al, 1997, Kitai et al, 2000).

Some of the viral proteins such as Tat, have been shown to stimulate the production of MCP-1, IL 8 and alpha chemokine 1P-10 in cultured astrocytes by a mechanism involving mitogen-activated protein kinase (MAPK) (Conant et al, 1998; Kutsch et al, 2000).

1.10.3. Selective vulnerability of neuronal subpopulations

It has been suggested that certain neuronal populations are more susceptible of damage during HIV infection. In particular those of the dopaminergic system. Loss of dopaminergic neurons has been suggested to underlie clinical deficits of this system observed in some individuals during the course of HIV infection. The clinical symptoms worsened when patients take drugs acting on dopaminergic system (Nath et al, 2000). In non-human primate models, deficit of the dopaminergic system

appears early during the asymptomatic period of SIV infection. In this model drugs which increase dopamine availability have been shown to increase the SIV encephalitic lesion and SIV replication (Czub et al, 2001). Extrapyramidal symptoms have been shown to be frequent in HIV infected patients and it has been recently suggested that they are related to neuronal degeneration of the substantia nigra (Itoh et al, 2000). Selective vulnerability of hippocampal neurons to HIV related injury has also been suggested (Petito et al, 2001).

In summary, early entrance of the HIV into the brain after infection is accepted and it is believed to be through the BBB in a process not well understood. The up-regulation of adhesion molecules on peripheral monocytes has been suggested. HIV primarily infects perivascular macrophages/microglial cells due to the expression of β chemokine receptors such as CCR3 and CCR5 on the cell surface of these cells. Infected macrophages and microglia can be activated and release cytokines, chemokines and other chemical mediators of inflammation which act on synaptic and dendritic terminals as well as on cells of the CNS. Infected CNS cells also shed some viral proteins, which in turn can activate a wide range of resident cells, favoring the release of more mediators and impairing their function. In the case of astrocytes, their impairment can generate excitotoxic damage to neurons and probably other cell types in the CNS. It is also accepted that most of the CNS damage occurs after the immunodeficiency takes place.

In recent years, after the introduction of combination anti-retroviral therapy an improvement in neurological function has been observed (Price et al, 1999). Some groups have suggested that combination therapy and more specifically HAART has less impact on HIV associated cognitive motor complex than for opportunistic

conditions, due to the poor penetration of these drugs into the CNS (Dore et al, 1999). However, other studies have shown that the neurological improvement is also reflected by reduction on the prevalence of HAD (Maschke et al, 2000). The incidence of neurological complications has declined since introduction of HAART, and improvement of the immune status of patients measured by CD4 counts has also been observed (Sacktor et al, 2001). As anti-retroviral therapies have lower concentrations in the CNS, it has been suggested that drug resistant mutants may develop in the CNS (Cavert and Haase, 1998). HAART has failed to completely eradicate HIV infection and in some cases HAART has also failed to suppress HIV replication. Thus, an increase in incidence and prevalence of neurological disease among HIV infected individuals seems possible (Sacktor et al, 2001).

1.10.4. Viral Factors

Certain viral characteristics are important for HIV infection of the CNS. Macrophage tropic strains of HIV have been shown to effectively infect microglia and it has been suggested that this tropism might be necessary for brain infection (Power et al, 1995). It has also been shown that brain-derived HIV strains differed from the viral stains obtained from spleen tissue of the same patient. Differences in *env* sequences between viral strains obtained from brain tissue of demented and non-demented cases have also been reported. This suggests that certain *env* sequences may account for the development of cognitive impairment (neurovirulence) (Power et al, 1995).

At present there is uncertainty about the origin and genetic evolution of brain derived HIV strains, since viral evolution (measured by the pattern of nucleotide substitution in the viral *env* gene and possibly other viral genes) has been shown to occur. This

positive selection has been shown to be secondary to the host immune response or anti-retroviral therapy. The genetic evolution of HIV is exemplified by the co-receptor usage. Escaped HIV mutants in response to anti-retroviral therapy and immune response of the host have all been shown to occur during the course of HIV infection. (Carrillo et al, 1998; Yang et al, 2000; Crandall et al, 1999, Shapshak et al, 1999).

1.11 Neuropathology

The neuropathological changes associated with HIV infection can be broadly divided into those caused directly by HIV infection and opportunistic conditions, which develop in some patients after the immunodeficiency takes place. Early neuropathological studies demonstrated significant histological changes including opportunistic conditions in an important number of cases, ranging from 74-88% (Anders et al, 1986; Lantos et al, 1989; Burns et al, 1991). In 1991 a consensus report proposed a neuropathology-based terminology for HIV- associated disease of the nervous system (Budka et al, 1991). Histological findings were defined with the aim of unifying the terms used for the description of these findings among different research groups.

1.11.1. HIVE

The term HIVE was morphologically defined as multiple, disseminated foci composed of macrophages, microglial cells and multinucleated giant cells. The later was considered the hallmark of HIVE if the other histological features were also

present. For the histological diagnosis of HIVE in absence of multinucleated giant cells, the identification of HIV antigens (by immunohistochemistry) or HIV nucleic acids (by in-situ hybridization) is necessary. These histological features are found more frequently in white matter, subcortical grey matter and cortex, in that order (Budka et al, 1991). Diffuse white matter lesions usually coexist with HIVE (Budka et al, 1991). The incidence of HIVE varies from place to place. It has been reported in 28% of the AIDS cases in New York (Petito et al, 1986), in 40% in Vancouver (Cornford et al, 1992) and 25% in the UK (Davies et al, 1997). Although HIVE has been reported as the most frequent post-mortem finding in the UK and other countries, significant differences in the frequency of HIVE between different cities (four European and two American) has also been shown (Davies et al, 1997, 1998). Comparisons between risk groups in the UK showed that HIVE occurs more frequently in drug users (Davies et al, 1997). In Edinburgh, Scotland, HIVE was diagnosed in 59% of the intravenous drug users in contrast to 15% of the MSM cases (Bell et al, 1996). Highly significant differences in the frequency of HIVE between different risk groups have been obtained not only in Edinburgh but in other centres as well (Davies et al, 1998). HIVE appeared to be the morphological substrate of HAD in different populations (Wiley et al, 1994; Bell et al, 1996). This association between HIVE and HAD is not observed in all patients. That is, a substantial number of cognitively impaired cases had no histological evidence of HIVE and vice versa. Although a clear explanation for these observations is lacking, it has been suggested that intrinsic host-viral interactions may explain some of these differences (Bell et al, 1996). It has been suggested that survival after the development of HIVE for a period

of time may be necessary for the establishment of cognitive impairment (Soontornniyomkij et al, 1998; Wiley et al, 1994).

1.11.2. White matter abnormalities

The most common feature that is found in the histological examination of the brain of AIDS cases without opportunistic conditions, are white matter abnormalities (Navia et al, 1986). White matter abnormalities including mylein loss, reactive gliosis, infiltration of the white matter by macrophages and microglial cells and occasionally lymphocytes as well as multinucleated giant cells, usually observed in a symmetrical fashion affecting both cerebrum and cerebellum are the defining criteria for HIV leukoencephalopathy as stated by the consensus report of 1991 (Budka et al, 1991). The diagnosis of HIV leukoencephalopathy in absence of multinucleated giant cells requires the identification of HIV antigens or HIV nucleic acids by techniques described above. Myelin breakdown products in the cytoplasm of infiltrating macrophages in the white matter are considered as evidence of myelin loss. Pallor on myelin-stained sections is also considered as an indirect indicator of myelin loss. When associated with other histological features of HIV leukoencephalopathy, this constitutes one of the commonest histological findings in the brains of cases with AIDS. Another white matter abnormality, which consists of vacuolar swelling of myelin usually containing macrophages, is also considered as a histological component of HIV leukoencephalopathy and is known as vacuolar myelinopathy. The term vacuolar leukoencephalopathy refers to more extensive vacuolar myelinopathy, accompanied by axonal swelling, macrophage infiltration, axonal loss and occasionally necrosis that may be either multifocal or diffuse in distribution

(Schmidbauer et al, 1990). This was also included in the 1991 consensus report as HIV infection specific white matter change (Budka et al, 1991). The frequent co-existence of HIVE and HIV leukoencephalopathy in some cases has generated some concern about the validity of this division (Everall and Lantos, 1991). In the pathogenesis of HIV leukoencephalopathy myelin loss has been implicated, but electron microscopy studies have failed to demonstrate demyelination in cases in which myelin pallor was observed. Instead indirect evidence of BBB abnormalities have been shown in these cases (Budka, 1991; Everall and Lantos, 1991). White matter abnormalities seen on MRI brain scans have been shown to correlate with gliosis and immunohistochemically stained extravascular plasma proteins in tissue sections (Power et al, 1993).

1.11.3. HIV associated myelopathy

In 1991, the American Academy of Neurology AIDS Task Force, included the HIV associated myelopathy within the group of severe manifestations of HIV-1 associated cognitive/motor complex and defined the clinical criteria for the diagnosis of this condition (Janssen et al, 1991). Morphologically, the HIV associated myelopathy frequently shows extensive vacuolation of the lateral and posterior columns of the spinal cord similar to the vacuolation observed in sub-acute combined degeneration caused by deficiency in cobalamine (vitamin B12). Thus, since 1985, this condition is known as vacuolar myelopathy (Petito et al, 1985). Isolation of HIV from spinal cord tissue and demonstration of HIV antigens on infiltrating macrophages and multinucleated giant cells suggesting productive HIV infection within the spinal cord (Maier et al, 1989) granted the inclusion of this entity among the HIV associated lesions of the CNS, although these findings were not observed in other studies

(Rosenblum et al, 1989). The consensus report in 1991 (Budka et al, 1991) defined vacuolar mylopathy as multiple vacuolar myelin swellings and macrophage infiltration affecting multiple areas of the spinal cord, predominantly in the dorsolateral spinal tracts. Macrophages, some of them inside the vacuoles, astrocytosis in occasional cases and in severe cases axonal degeneration are all characteristic of this condition (Petito et al, 1985, 1986; De Girolami et al, 1990; Budka, 1991). These vacuoles vary in size from 10 to 50μ and may be extensive. Lesions are symmetric without confinement to anatomical tracts. The thoracic segment of the spinal cord appears to be the most affected (Petito et al, 1986; Tan et al, 1995). The most prominent clinical feature is a progressive course of leg weakness, spastic or ataxic sensory disturbances and incontinence (Dal Pan et al, 1994). The reported frequency of vacuolar myelopathy in post-mortem series varies widely. It was diagnosed in 3.3% of 89 cases in California (Anders et al, 1986), in 17% of 138 cases in Paris (Henin et al, 1992), in 20% of 41 cases in Edinburgh (Shepherd et al, 1999), in 29% of 153 cases in New York (Petito et al, 1986) and in 46% of 215 cases in Baltimore (Dal Pan et al, 1994). The pathogenic role of HIV in the development of vacuolar myelopathy is controversial, since HIV antigens and HIV DNA have been observed in infiltrating macrophages and multinucleated giant cells, but no correlation between vacuolar myelopathy or with the severity of the histological findings has been reported (Petito et al, 1994; Tan et al, 1995, 1996; Shepherd et al, 1999). Similarly, no correlation between HIV-1 RNA levels in CSF and plasma and severity of vacuolar myelopathy as measures by electrophysiological tests of nerve conduction has been observed (Geraci et al, 2000).

The presence of microglial nodules, macrophages, and/or multinucleated giant cells expressing HIV antigens in either or both white or grey matter of the spinal cord are the histological characteristics of HIV myelitis. Occasional perivascular lymphocytes complete the histological picture (Henin et al, 1992). Lack of correlation between HIV myelitis and vacuolar myelopathy has been reported by different groups (Henin et al, 1992; Petito et al, 1994). It has been shown that when both conditions were present in the same case, infected cells were not found in areas affected by vacuolar myelopathy (Henin et al, 1992). In Edinburgh, HIV myelitis was reported in 10% of 74 cases and it was seen significantly more frequently in drug users than in MSM cases. In contrast, the frequency of vacuolar myelopathy was almost similar between these two risk groups (Shepherd et al, 1999). Finally, vacuolar myelopathy may also occur in diseases unrelated to HIV infection.

Thus, all the above suggests that vacuolar myelopathy may not be pathogenically linked to HIV infection. However, vacuolar myelopathy is frequently seen in cases with HIV (Henin et al, 1992; Petito et al, 1994), and has been found significantly associated with a number of opportunistic conditions. This suggests that the development of this condition is related to the severity of immunosuppression (Dal Pan et al, 1994). Recently, clinical remission of HIV myelopathy after HAART has been shown, and correlated with a dramatic increase of CD4+ T cells count, supporting the role of the immune system in the development of vacuolar myelopathy (Staudinger and Henry, 2000). The identification of macrophages in the posterior and lateral columns of the spinal cord even in HIV negative controls and the increased number of these cells in HIV positive cases with and without vacuolar myelopathy suggest that these cells precede the development of vacuolar myelopathy

(Tyor et al, 1993). Immunohistochemically, identification of TNFα positive macrophage/microglial cells in the areas affected by vacuolar myelopathy and increased levels of this cytokine in the CSF of cases with this condition suggest that TNFα may be relevant in the pathogenesis of vacuolar myelopathy (Tan et al, 1996; Geraci et al, 2000).

In summary, the pathogenesis of vacuolar myelopathy is still unclear, but immunosuppression and probably release of cytotoxic and myelinotoxic substances by infiltrating macrophages may all be related to its development.

1.11.4. Vascular lesions

A number of cerebrovascular abnormalities have been described in AIDS cases. These include; subarachnoid haemorrhages, subdural haematomas, cerebral infarcts, intracerebral haemorrhage, calcification of the blood vessels, mural thickening of small blood vessels and vasculitis. In adults, cerebrovascular abnormalities have been reported in 8-34% of autopsy series (Anders et al, 1986; Mizusawa et al, 1988; Lantos et al, 1989; Berger et al, 1990). In children, the frequency of cerebrovascular abnormalities (especially cerebral infarction and vasculitis) appears to be higher. Cerebral infarction and cerebral vasculitis has been reported in up to 30% each in HIV positive children (Atwood et al, 1993; Moriarty et al, 1994). In Edinburgh, cerebral infarction in adults with AIDS was found in 5.5% of 26 cases without opportunistic conditions or cardiovascular sources of emboli. HIV positive drug users and haemophiliacs appear to have increase incidence of cerebral infarcts and intracerebral haemorrhage (Esiri et al, 1989; Pinto, 1996). Small, usually multiple, cerebral infarcts affecting the basal ganglia are the most frequent finding in AIDS

cases. These small infarcts are usually clinically silent and are often associated with thickening of small blood vessel walls, calcification and perivascular inflammatory infiltrates (Pinto, 1996; Connor et al, 2000). Intracerebral haemorrhage has been found frequently associated with thrombocytopenia, and primary CNS lymphoma, while cerebral infarcts are often associated with non-bacterial thrombotic endocarditis or opportunistic infections (Pinto, 1996). Vasculitis has been implicated in the pathogenesis of cerebral infarcts in HIV infected adults but a direct pathogenic link with HIV has not been clearly shown. An immunologically mediated vascular damage seems to be the most likely cause in AIDS cases (Pinto, 1996). *In vivo*, cerebrovascular reserve capacity and blood flow velocity measured by transcranial doppler sonography, have been shown to be significantly reduced in HIV positive cases compared with age matched controls. These parameters were even more reduced in AIDS cases than in asymptomatic HIV positive patients. Alterations of cerebral resistance at the arteriolar level were suggested as the cause of these alterations but the clinical significance of these findings has not been yet clear (Brilla et al, 1999).

1.11.5. Lymphocytic meningitis

After the primary infection, around 4% of HIV individuals develop aseptic meningitis (Budka, 1991). Lymphocytic meningitis is generally regarded as a characteristic finding in early HIV infection. It was reported in up to 74% of 23 HIV positive pre-symptomatic drug users (Bell et al, 1993). Morphologically, a lymphocytic infiltrate of the leptomeninges of varied severity, usually spreading to perivascular spaces of the brain is observed (Budka et al, 1991). Oher studies

however, have reported this histological feature in all stages of HIV infection, but a significantly higher frequency of this condition is found in HIV positive pre-symptomatic cases (Kibayashi et al, 1996). The lymphocytic infiltrate consists almost entirely of CD8+ T lymphocytes, although occasional CD4+ T lymphocytes and B lymphocytes have been reported (Tomlinson et al, 1999, Bell et al, 1993). The association of this condition with HIV is not clear (Budka et al, 1991a), and no apparent opportunistic condition has been reported associated with lymphocytic meningitis (Budka et al, 1991).

1.11.6. Peripheral nerve pathology

Peripheral nerve abnormalities can be present in various stages of HIV infection. Their relation to HIV is not clear and the consensus report in 1991 suggested a description, similar to clinical terms, for the abnormalities of the peripheral nervous system seen in HIV infected individuals. Lack of specific HIV changes in most cases makes these peripheral nerve abnormalities similar to those observed in HIV negative individuals (Budka et al, 1991).

1.12 Treatment

The first clinically approved drug for the treatment of HIV infection was Zidovudine (AZT). This drug acts as a reverse transcriptase inhibitor and belongs to the generic group of nucleoside analogue reverse transcriptase inhibitors (NRTIs). In the late 1980's, monotherapy with this drug was the predominant treatment for HIV infected individuals. Treatment with AZT was shown to decrease mortality and frequency of

opportunistic conditions (Fischl et al, 1987). Decreased sensitivity to AZT after short-term use and development of resistant HIV strains in patients receiving monotherapy were soon described (Larder et al, 1989; Tudor-Williams et al, 1992; Montaner et al, 1993, D'Aquila et al, 1995). However, due to the rapid HIV suppression of AZT, which has been reported to reach maximum suppression by day seven (Loveday et al, 1995), it has been recommended for short-term use in situations such as pregnancy. AZT significantly reduces the risk of vertical HIV transmission when administered prior and intra-partum to the mother and for 6 weeks to the newborn (Connor et al, 1994; Lallemant et al, 2000). AZT and other NRTIs enter cells by diffusion and are intracellularly phosphorylated and incorporated into the HIV DNA, stopping DNA synthesis acting as chain terminators. Other NRTIs include Didanosine, Zalcitabine, Stavudine, Lamivudine and Abacavir. Additive and/or synergistic antiviral effects have been shown by combination of 2 NRTIs. Slow HIV disease progression and improved survival were reported (Hammer et al, 1996), but the development of multi-drug resistance was also observed (Larder et al, 1995).

Also in the late 1980s, other antiretroviral compounds were developed the non-nucleoside reverse transcriptase inhibitors (NNRTIs). There are 3 such compounds approved by Food and Drug Administration in North America namely, Nevirapine, Delavirdine, and Efavirenz. These drugs have a different mechanism of action and pattern of resistance from NRTI. The mechanism of action of these drugs is mediated by the binding of these compounds near the viral polymerase catalytic domain. The combination of two NRTI with one NNRTI demonstrated improvement of antiviral and immunological effects (Floridia et al, 1999; Montaner et al, 1998), although the

use of Nevirapine (the first of NNRTIs approved for HIV treatment) was associated with frequent hypersensitivity reactions (Carr et al, 1996).

The third group of drugs used for HIV infection treatment is the protease inhibitors (PI). These compounds mediate the inhibition of the processing of Gag and Gag-PoL protein precursors, preventing the maturation of virions. Viral mutations have also been reported in association with PI treatment (Markowitz et al, 1995). Five such drugs are approved in North America: Saquinavir, Ritonavir, Indinavir, Nelfinavir, and Amprenavir. Combined use of two or three of these drugs has been shown to induce persistent suppression of HIV. A combination of three drugs (usually two NRTIs and one PI), has been shown to significantly slow the progression of HIV disease, even in patients with low CD4 counts (Hammer et al, 1997). The combined use of at least three anti-retroviral drugs is known as highly active anti-retroviral therapy (HAART) and it is now the standard therapy in developed countries. HIV eradication based on achievable HIV suppression is the scientific rationale behind HAART. The presence of latently infected cells has created a great concern about the feasibility of this goal (Perelson et al, 1997) and it has been suggested that active viral replication occurs *in vivo* in patients receiving HAART, due to the presence of unintegrated HIV DNA in resting T cells (Chun et al, 1997a; Zhang et al, 1999). The benefits of HAART include decline in morbidity and mortality due to AIDS and arrested decline of CD4 T cell counts, and reduction in plasma HIV RNA load and immune activation (Palella et al, 1998; Detels et al, 1998).

Adherence to HAART regimes appears to be necessary for obtaining optimal results, but the number and timing of doses, the number of pills and some food restrictions, apart form the adverse effects of some of these compounds make compliance

difficult. Commonly recommended treatment regimes and monitoring strategies have been recently published (Carpenter et al, 2000).

More recently some immunological therapies in association with HAART have shown promising results. Among these, the administration of IL 2 has been shown to increase CD4 cell counts and decrease the viral load to a greater extent than HAART alone (Davey et al, 2000).

1.12.1. Adverse effects of treatment

1.12.1.1 Mitochondrial Toxicity

Mitochondrial toxicity has been observed in patients receiving reverse transcriptase inhibitors (RTI) and monophosphorylated nucleotide analogue RTI (NtRTI). The metabolism of these compounds involves intracellular phosphorylation to active triphosphate forms, which are incorporated into the DNA synthesised by HIV, abruptly stopping DNA synthesis. The suggested mechanism of toxicity for these drugs is the inhibition of DNA polymerase gamma, which in medium and long term lead to mitochondrial dysfunction and cellular toxicity (Kakuda, 2000) This form of mitochondrial dysfunction shares similarities with some inherited mitochondrial diseases.

1.12.1.2. Hypersensitivity

Hypersensitivity mainly to non-nucleoside reverse transcriptase inhibitors is seen in up to 20% of the patients receiving these compounds. Hypersensitivity is manifested by an erythematous maculopapular pruritic and confluent rash, which is usually

preceded by constitutional symptoms. Increased frequency of cutaneous reactions to a number of drugs including aminopenicillins and trimetroprin-sulfametoxazole have been well-documented in HIV positive cases (Bigby et al, 1986; Coopman et al, 1993). The pathogenesis of hypersensitivity reactions is unknown (reviewed in Carr and Cooper, 2000).

1.12.1.3. Lipodystrophy

Lipodystrophy syndrome was first described in 1998 as a complication of protease inhibitor treatment and was manifested by peripheral lipodystrophy, hyperlipidaemia and insulin resistance (Carr et al, 1998). Fat wasting of the face, limbs and upper trunk is accompanied by metabolic abnormalities which include high triglyceride, cholesterol and insulin levels in plasma and weight loss in men (Carr et al, 1998), but weight gain and increased abdominal and breast size have been observed in women (Dong et al, 1999). Most of the metabolic abnormalities, mainly the lipid and lipoprotein abnormalities, reverse after discontinuation of HAART, suggesting a direct drug effect (Hatano et al, 2000). The lipoprotein abnormalities reported in association with HAART include significantly higher levels of plasma very low density lipoprotein (VLDL) cholesterol, low density lipoprotein (LDL) cholesterol, intermediate density lipoprotein (IDL) cholesterol, and lipoprotein (a) (Lp(a)) (Koppel et al, 2000; Purnell et al, 2000). Some of these lipoprotein abnormalities and especially the high levels of Lp(a) are risk factors for coronary heart disease. Dyslipidemia at concentrations associated with coronary heart disease occurs in a frequency reaching 70% of the cases with lipodystrophy. Other abnormalities observed associated with lipodystrophy include insulin resistance, lactic acidaemia,

liver dysfunction and type 2 diabetes mellitus, which has been reported in about 8-10% of the cases (Reviewed in Carr and Cooper, 2000; Vigouroux et al, 1999a, 1999b).

The pathogenesis of this syndrome is obscure but an inhibition of lipid and adipocyte regulatory proteins, which have homology to the HIV protease, has been suggested. Mitochondrial toxicity of NRTIs has also been suggested (Reviewed in Vigouroux et al, 1999b), since lipodystrophy has also been shown to be associated with NRTI (Mulligan et al, 2001; Saint-Marc et al, 1999).

Other suggested pathogenic mechanisms include imbalances in dehydroepiandrosterone and cortisol. Serum cortisol levels have been found increased and serum levels of dehidroepiandrosterone have been found significantly lower in patients with lipodystrophy when compared with normal controls. Furthermore, insulin levels have been shown to inversely correlate with endogenous gonadal levels of dehidroepiandorosterone in HIV positive men (Christeff et al, 1999). These findings are of interest since steroid hormones are involved in adipocyte homeostasis. Prospective studies and clear diagnostic criteria will help to elucidate the pathogenesis of this syndrome.

1.12.1.4. Effects on CD8+ T cells

Finally, another possible side effect of HAART is related to the reduction of non-cytotoxic suppression of HIV replication by CD8+ T cells. After suppression of HAART therapy, low viral loads were maintained and the reduction of CD8 antiviral response was not observed. It has been suggested that antigenic challenge is required

for maintaining the anti-HIV, CD8 mediated immunity or that HAART has some inhibitory effects on CD8 function (Stanford et al, 2001).

Chapter 2

APOLIPOPROTEIN E

Apolipoproteins are synthesized primarily by the liver, although other organs such as the intestine and brain also synthesize them (see below). The function of apolipoproteins in the modulation of lipoprotein metabolism is based on interactions of these protein moieties with cell surface receptors of the low density lipoprotein (LDL) receptor family. Differential receptor binding affinity of different apolipoproteins mediates lipoprotein metabolism.

2.1. Lipids, lipoproteins and apolipoproteins overview

Lipids are found throughout the body in cell membranes and are insoluble or minimally soluble due to their hydrophobic composition. This poor solubility allows the maintenance of cell integrity and the separation of organelles in cell cytoplasm. They are substrates for hormonal production (gonadal and adrenal steroids), and an important source of energy. They act as intracellular and extracellular messengers (for a review see Mahley et al, 1998). Lipids can be divided in several classes namely fatty acids, triglycerides, cholesterol and phospholipids. They are transported throughout the bloodstream by macromolecular complexes known as lipoproteins or bound to albumin in a non-esterified state (free fatty acids).

Lipoproteins are spherical particles with a core of mostly hydrophobic lipids and a surface layer of more hydrophilic constituents such as proteins (apolipoprotiens), free cholesterol and phospholipids (reviewed in Mahley et al, 1998). They serve as vehicles to transport fat soluble vitamins, drugs, antioxidants enzymes and viruses in the blood (reviewed in Mahley et al, 1998). Lipoproteins can be differentiated into several classes based on the physical-chemical characteristics such as lipid content, density and apolipoprotein composition.

Apolipoproteins are protein moieties found in the surface of lipoproteins in plasma and are given letter designations.

2.2. Lipid Classes

2.2.1. Fatty Acids

Fatty acids are an available source of energy, they can be obtained from the diet and a wide range of food sources provides different types of fatty acids. They may be transported in blood as free fatty acids, bound to albumin or they can form macromolecular complexes and be transported by lipoproteins. Depending on their length and the number and position of the double bonds, the fatty acids can be divided into:

1) Saturated, in which all the carbon atoms have a complementary number of hydrogen atoms and no double bonds.

2) Unsaturated, which in turn can be subdivided in:

Monounsaturated, when only one double bond is found.

Polyunsaturated when more than one double bond is found. Polyunsaturated fatty acids can be subdivided as well into Omega 6 fatty acids (e.g. Linoleic acid, arachidonic acid) and Omega 3 fatty acids.

Fatty acids can be used as an energy source or they can be found as a constitutive part of triglycerides and phospholipids after esterification to glycerol. When esterified to a hydroxyl group they can be found in the cholesterol molecule. Fatty acids are essential for prostaglandin synthesis.

2.2.2. Cholesterol

Cholesterol is synthesized in most tissues by the enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase. Cholesterol is a major component of cell membranes. It is also a precursor of bile acids (which are necessary for the absorption of fat in the intestine lumen) and steroid hormones. Two thirds of the cholesterol in blood is esterified. Cholesterol is the major core lipid of low-density lipoproteins (LDL) found mainly as cholestryl esters. Cholesterol can also be found in the surface of lipoproteins as free cholesterol, which could be esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT). This reaction can take place in plasma and allows cholesterol to move into the core of the lipoprotein molecule. Other lipoproteins such as chylomicrons and very low density lipoprotein VLDL contain a low proportion of cholesterol (7% and 15% respectively). In type III hyperlipoproteinemia, chylomicrons and VLDL remnants (remnants refers to abnormal the constitution of lipoproteins in their lipids or apolipoprotein content), have a high cholesterol content and therefore these

lipoproteins have a β electrophoretic motility rather than the normal pre-β. For this reason these lipoproteins are referred collectively as β-VLDL (Mahley and Rall, 1995)

Cholesterol cannot be catabolized, but is secreted as bile acids into the intestine or excreted as free cholesterol in the bile. Half of the secreted cholesterol and up to 97% of the excreted cholesterol is reabsorbed in the intestine; this modulates the synthesis of cholesterol and bile acids in the liver. (Mahley et al, 1998).

Cholesterol is either absorbed from the diet or synthesized by cells in the body. Cholesterol is synthesized in the liver, skin, adrenals, intestine and brain. Biosynthesis of cholesterol is regulated by HMG-CoA reductase. The activity of this enzyme is back regulated by the cholesterol content of cells.

2.2.3. Triglycerides

Triglycerides are complex lipids, which consist of three fatty acid molecules esterified to a glycerol molecule. Triglycerides are the major lipid type found in chylomicrons and VLDL (90% and 80% respectively). When triglycerides are hydrolyzed in the liver, muscle, and adipocytes or in lipolipoprotein particles, they liberate the fatty acid chains to be used as an energy supply. Hydrolysis of triglycerides can occur in plasma by enzymes such as lipoprotein lipase (LPL) or hepatic lipase (HL). Exchange of triglycerides for cholesteryl esters between chylomicrons and VLDL and cholesterol containing lipoproteins such as LDL and high-density lipoproteins (HDL) is mediated by cholesteryl ester tranfer protein (CEPT).

2.2.4. Phospholipids

Phospholipids are the most hydrophilic of all the lipids. Their unique combination of hydrophobic and hydrophilic regions makes them an essential component of cell membranes and the major component of the lipoprotein coats. Phospholipid structure consists of two fatty acid chains esterified to two hydroxyl groups of glycerol. A phosphate is esterified to the third hydroxyl group of the glycerol molecule, to form phosphatidic acid.

Phosphatidic acid can be esterified to choline (phosphatidyl choline) or to serine (Phosphatidyl serine) which are hydrophilic molecules.

2.2.5. Lipoproteins

Lipoproteins are macromolecules, which transport hydrophobic lipids throughout the body. Different lipoproteins have different composition of lipids and proteins. Lipids transported by lipoproteins include triglycerides, cholesteryl esters, phospholipids and free cholesterol. Protein moieties found in the surface of lipoproteins are known as apolipoproteins. Six different types of lipoproteins are found in plasma and their differentiation is based on their physical-chemical characteristics such as density, electrophoretic mobility, lipid content and apolipoprotein content (Mahley et al 1998).

2.3. Apolipoproteins

2.3.1. Apolipoprotein A (ApoA)

Apolipoprotein A (all isoforms) are synthesized in the liver and intestine. There are three different forms of ApoA designated as ApoAI, ApoAII and ApoAIV. ApoAI, AIV and ApoCIII are encoded in the long arm of chromosome 11, in the region 11q 23-24.

ApoAI

ApoAI is synthesized by liver and intestine and comprises up to 80% of HDL protein content. ApoAI is also found in chylomicrons, due to the intestine secretion. Mature circulating ApoAI is 243 amino acids long. Apart from being a major structural protein for HDL, ApoAI can activate the enzyme responsible for the cholesterol esterification, lecithin:cholesterol acyltranspherase LCAT on the surface of HDL. This function is also present in ApoAIV and ApoCI.

ApoAI has been implicated in the reverse cholesterol transport pathway due to its action of cholesterol acceptor, when cholesterol is released from cells. It has a binding domain for scavenger receptor B1 (Ginsberg, 1998) which is a HDL receptor, mediating the cholesterol delivery to the cells. ApoAI protects against the severe atherosclerosis observed in ApoE knockout mice (Mahley et al, 1998).

ApoAII

ApoA II is the second most common ApoA in the plasma. It is encoded by a gene in chromosome 1 at position q 21-23 and is synthesized in the liver. The mature circulating form is 77 amino acids long. ApoAII is found as a structural protein

component of HDL. ApoAII usually forms homodimers and heterodimers with ApoE, which only occur in individuals with ApoE *ε2* or *ε3* alleles.

ApoAIV

ApoAIV is mostly found as free apolipoprotein in the circulation, although it can be a minor structural protein component of HDL and chylomicrons. ApoAIV may activate LCAT enzyme or it may be a necessary condition for the activation of LPL by HDL-ApoCII.

2.3.2. Apolipoprotein B (ApoB)

Apolipoprotein B (ApoB) is synthesized in liver. Two forms of ApoB can be found. Both of them are encoded by a single gene in chromosome 2 in the region p 23-24. These forms are known as ApoB100 and ApoB48. A particular and as yet unknown mRNA editing mechanism is responsible for this variation: ApoB100 consist of 4536 amino acids while ApoB48 only has 2152 amino acids. A single nucleotide substitution, cytosine for uracyl is responsible for the difference in length observed between these two forms of ApoB. ApoB48 lacks of the carboxyl-terminal domain of ApoB100 and it is in that region in which the LDL receptor ligand resides. Therefore ApoB48 has no receptor-binding domain. ApoB100 is present in VLDL, intermediate density lipoproteins (IDL). It is the only Apolipoprotein found in LDL and it is responsible for the clearance of LDL from the circulation. Apolipoprotein E (ApoE) is the apolipoprotein responsible for the clearance of VLDL and IDL.

2.3.3. Apolipoprotein C (ApoC)

Three major forms of ApoC are present in plasma namely, ApoCI, ApoCII and ApoCIII. All of them are synthesized by the liver and all are structural protein components in various proportions of chylomicrons, VLDL, IDL, and HDL. ApoCI and CII, are encoded by different genes in chromosome 19 close to the gene encoding ApoE in the region 19q 13.2. ApoCIII is encoded by a gene cluster on chromosome 11, which also encodes ApoAI and ApoAIV.

Apolipoprotein C seem to regulate triglyceride metabolism due to the active exchange of ApoC apolipoproteins from cholesterol rich isoforms of HDL to VLDL, IDL and chylomicrons (triglyceride rich lipoproteins). ApoCI and CIII interfere with ApoE/receptor interaction on endothelial cell surfaces (probably by displacement), which is necessary for LPL hydrolysis of triglycerides. ApoCII is a cofactor for LPL activation.

2.3.4. Apolipoprotein E (ApoE)

ApoE is a constitutive protein of several different lipoproteins such as chylomicrons (a subclass of HDL), chylomicron remnants, VLDL, IDL, and a subclass of HDL known as ApoE containing HDL or HDL1. ApoE modulates the metabolism of lipoproteins and lipids by mediating the binding of lipoproteins to LDL receptors or to other LDL receptor family receptors. Additionally, ApoE seems to be involved in cholesterol metabolism and transport from cell to cell, delivering cholesterol to cells undergoing proliferation and repair. ApoE also seems to play a role in smooth muscle proliferation and lymphocyte activation.

ApoE is a 34KDa protein, composed of 299 amino acids. It is encoded by a single gene in chromosome 19 in the apolipoprotein cluster region 19q 13.2. The ApoE gene is 37Kb in length and contains four exons and three introns. It is closely related to ApoCI and CII genes (Davignon et al, 1988). Similarities (especially as regards to the intron locations) between ApoE gene and other Apolipoprotein genes (ApoC) suggest a common origin.

Different types of cells in the liver, brain, spleen, lungs, adrenal glands, ovary and kidney in different animal species produce ApoE. In the liver, ApoE is synthesized by parenchymal cells. In other organs it is synthesized by macrophages and smooth muscle cells. This heterogeneity in ApoE synthesizing cells and tissues underlies the importance of ApoE in lipid transport and probably in other unrelated functions (reviewed in Mahley, 1988). High levels of ApoE are found in interstitial fluid and lymph especially associated with lipoproteins. In plasma, levels of ApoE vary from 30-70μg/ml. The liver produces 75% of this. In brain, up to a third of this amount is produced, and astrocytes are the major source of this protein in the CNS. The brain contains the second highest levels of ApoE (after the liver) and in the CSF, the levels of ApoE are 5-10% of the plasma levels. ApoE is a polymorphic protein. A single gene with three different alleles known as *ε2*, *ε3*, and *ε4* encodes apolipoprotein E. A substitution of a single amino acid at positions 112 and 158 of the protein is responsible for the three different protein isoforms. These isoforms are universally known as E2, E3, and E4. The most common of these isoforms is the E3. A substitution of arginine for cysteine at position 158 is found in ApoE E2. In ApoE E4 a cysteine is substituted by an arginine at residue 112. Therefore the allele *ε2* encodes an ApoE with two cysteine amino acids and position 112 and 158, while the

ε4 allele encodes an ApoE with two arginine amino acids in the above mentioned positions (Mahley, 1988; Mahley and Rall, 1995; Mahley et al, 1998). These amino acid substitutions of the neutral cysteine for basic arginine renders E4 more basic and confers on it an extra positive charge when compared to E3 (Mahley and Rall, 1995; Mahley et al, 1998; Davignon et al, 1988).

The allelic variations give rise to six different genotypes with three homozygous forms (*ε2/ε2, ε3/ε3, ε4/ε4*) and three heterozygous forms (*ε2/ε3, ε2/ε4, ε3/ε4*). Each allele is inherited in a co-dominant manner (Davignon et al, 1988; Mahley, 1988; Mahley and Rall, 1995; Mahley et al, 1998; Orht et al, 1996).

After the characterization of the ApoE structure, a precise method for the genotyping of the different isoforms has been used, based on polymerase chain reaction. It is mediated by the action of a restriction endonuclease such as HhaI, which recognizes the sequence guanine-cytosine-guanine-cytosine (GCGC) which is absent in two positions in ApoE *ε2* in one in *ε3* and it is present in two positions in ApoE *ε4*. In this way fragments of different size are produced after the initial amplification of the exon four encoding the polymorphic fragment of the ApoE. The different fragments can be visualized using various gel electrophoresis (Hixson and Vernier, 1990; Kontula et al, 1990; Wenham et al, 1991; Gioia et al, 1998).

2.3.4.1. ApoE function

The carboxyl-terminus of ApoE is the major lipid-binding site of ApoE as in all other Apolipoproteins and the amino-terminal fragment of ApoE contains the receptor-binding and heparin/proteoglycan-binding domains. The amino-terminal fragment of ApoE consists of four helices arranged in anti-parallel fashion. The receptor binding

and heparin/proteoglycan-binding regions lie on helix 4. Substitution of the cysteine for arginine at position 158 in ApoE E2 significantly lowers the positive ion potential of the receptor-binding domain, almost abolishing the ability of ApoE E2 to bind the LDL receptors, but does not affect its heparin/proteoglycan binding ability. In contrast to the effect of amino acid substitution at position 158, the amino acid substitution at position 112 in ApoE E4 does not alter the receptor-binding activity, but changes the helix arrangement. The carboxyl-terminus domain of ApoE is critical for triglyceride-rich lipoprotein binding. ApoE E2 and E3 bind preferentially to HDL, while ApoE E4 binds to VLDL possibly due to its abnormal helix orientation (Mahley and Rall, 2000).

Detection of Apolipoprotein E polymorphism by PCR and restriction endonuclease (Hhal).

	Amino acid position							
	112			**158**				
ε2	TGC			TGC				
____	_____	**91**		_____	**82**	____		
ε3	TGC			CGC				
____	_____	**91**	_____	**48**	_____	**34**	____	
ε4	CGC			CGC				
____	_____	**72**	**19**	_____	**48**	_____	**34**	____

The fragment sizes are shown in base pairs. TGC: Cysteine, CGC: Arginine. The restriction endonuclease HhaI recognizes the sequence CGCG, which is absent in two positions in the ApoE *ε2* allele and it is present at both positions in the ApoE *ε4* allele.

The receptor binding and heparin/proteoglycan-binding regions lie on helix 4. Substitution of the cysteine for arginine at position 158 in ApoE E2 significantly lowers the positive ion potential of the receptor-binding domain, almost abolishing the ability of ApoE E2 to bind the LDL receptors, but does not affect its heparin/proteoglycan binding ability. In contrast to the effect of amino acid substitution at position 158, the amino acid substitution at position 112 in ApoE E4 does not alter the receptor-binding activity, but changes the helix arrangement. The carboxyl-terminus domain of ApoE is critical for triglyceride-rich lipoprotein binding. ApoE E2 and E3 bind preferentially to HDL, while ApoE E4 binds to VLDL possibly due to its abnormal helix orientation (Mahley and Rall, 2000).

There are racial differences in ApoE allelic frequencies and therefore in the ApoE phenotype. The ApoE E3/3 phenotype appears to be the most common among various populations. Its frequency varies from 50-70%, and it correlates with the *ε3* allelic frequency of 50-90%. *ε4* allele is the second most common and it accounts for 5-35% while *ε2* allele is the least frequent with a frequency of only 1-15% (Mahley and Rall, 2000). Although, ethnic differences do occur, it seems that the *ε3* allele is the most frequently found allele in every population except the Huli aboriginal group of the highlands of Papua New Guinea, in whom the most common is the *ε4* (Mahley et al, 1995; Davignon et al, 1988). Interestingly Chinese and Japanese populations have a higher frequency of *ε3* alleles than Caucasians (85% vs 79%) and Africans have a higher frequency of *ε4* than Caucasians (30% vs 15%) (Davignon et al, 1988; Mahley et al, 1995). In Caucasians, the ApoE *ε3* allele is found in approximately 77%, the *ε4* in 15% and the *ε2* in around 8% of the population. As each individual

inherits one allele from each parent, there are six phenotypes in the general population i.e. 3\3, 3\4, 3\2, 4\4, 4\2, and 2\2. The frequency for these in Caucasians is 55%, 25%, 15% for the first three respectively and 1-2 % for each of the rest (Mahley and Rall, 2000).

The *ε3* is considered the parent allele while the other two are considered variants (Mahley and Rall, 2000).

2.3.4.2. Lipid metabolism and ApoE

Lipoproteins such as VLDL and chylomicrons, which contain both cholesterol and triglycerides, also contain ApoE. In the circulation both lipoproteins become enriched with ApoE. In endothelial cells LPL hydrolyzes triglycerides releasing fatty acids for utilization by these cells, but this also occurs in other extrahepatic tissues. In the liver, triglycerides can be metabolized or resecreted with VLDL and cholesterol is then eliminated by the bile.

Lipolysis of triglycerides can be modulated by the amount of ApoE present in the lipoprotein surface. Thus accumulation of ApoE significantly reduces the rate of triglyceride lipolysis. This is of importance due to the isoform specific affinity for triglyceride rich lipoproteins (such as VLDL) being ApoE *ε4* the isoform that preferentially enriches these lipoproteins. The lipid delivery function of ApoE is mediated by the high affinity of some ApoE isoforms for the LDL receptor. Another pathway which is frequently used for ApoE is that mediated by heparansulphate proteoglycan (HSPG)/LDL receptor related protein (LRP). In this pathway, ApoE binds HSPG in the cell surface and can be lipolyzed by lipases. The ApoE-containing

lipoprotein can then be transferred to LRP for internalization or it can be internalized as part of the HSPG/LRP complex (Mahley and Rall, 2000).

Another of the functions of ApoE is to mediate the efflux of cholesterol from cells, which is mediated by ApoE-containing HDL. ApoE influences lipid levels in plasma in an isoform specific manner. Thus, ApoE E2 is associated with high levels of ApoE and triglycerides and low levels of ApoB and cholesterol. ApoE E4 is associated with increased levels of ApoB and cholesterol and low levels of ApoE when compared to the ApoE E3 (Gregg et al, 1986). Thus, the maximum ApoE levels are observed in individuals with 2\2 phenotype and the lowest in 4\4 phenotype. The levels of ApoB follow the inverse pattern (Mahley and Rall, 2000).

ApoE phenotype directly affects lipid metabolism and it is widely accepted that hypertriglyceridemic subjects have an over-representation of the *ε2* allele while hypercholesterolemic individuals have a higher frequency of *ε4* allele. Conversely *ε2* homozygous have lower levels of cholesterol in plasma than any other phenotype (Davignon et al, 1988; Mahley et al, 1998). It seems that the presence of one *ε4* allele increases the plasma levels of cholesterol. Hypertriglyceridemia is associated with the presence of at least one *ε2* allele possibly due to a defective lipolysis of triglyceride rich lipoproteins (chylomicrons, chylomicron remnants and VLDL remnant). The above mentioned observations are homogeneous among different racial populations and apply even in the presence of specific metabolic situations such as Diabetes mellitus (Dallongeville et al, 1992; Mahley et al, 1998).

Another interesting finding is that levels of ApoE also vary among different ApoE phenotypes; ApoE E4 is catabolized faster that any other isoform, having approximately the plasma half life of ApoE E3.

Several probable explanations can account for this difference. Structurally ApoE E4 has more positive charges that ApoE E3 or E2. This positive electric charge may prevent ApoE E4 from heterodimerizing with other apolipoproteins. In that way its catabolism may be accelerated (Gregg et al, 1986). Other possible explanations are related to the ApoE affinity for VLDL lipoproteins and for receptors of the LDL family. Individuals with *ε4* alleles have lower plasma ApoE levels than *ε3* positive carriers (Gregg et al, 1986).

As discussed earlier, differences in lipid metabolism exist among different ApoE phenotypes. These differences may be responsible for the increased risk of developing coronary artery disease and AD among *ε4* positive individuals (Davignon et al, 1988) as well as the ApoE role in modulation CNS response to injury, which will be discussed later.

2.4. ApoE and Immunity

2.4.1. ApoE and lymphocytes

Early studies in lipid metabolism showed that LDL derived from the plasma of patients with hepatitis B was able to suppress certain lymphocyte T cells functions such as E rosette formation (the formation of rosettes when lymphocytes are in contact with sheep red cells) (Chisary and Edgington 1975, Curtis and Edgington 1976). Other studies showed the presence of LDL receptors on lymphocytes, which are modulated by cholesterol or cholesterol-containing lipoproteins such as LDL (Ho et al 1976, Ho et al, 1977). Further work also demonstrated an inhibitory function of lipoproteins on lymphocyte proliferation (Morse et al, 1977). Activation of

lymphocytes after mitogen challenge was shown to be suppressed by LDL binding to the cell surface receptors, since it was abolished by heparin (which displaces membrane bound LDL) and by inhibition of endocytosis of LDL by low temperature. The existence of an immunoregulatory receptor on lymphocyte cell surfaces was proposed and the role of apolipoprotein content of LDL was also suggested to be important in modulating lymphocyte activation (Hui and Harmony, 1980). The inhibition of activation of lymphocytes by LDL was reversed by addition of transferrin to the culture media. Transferrin also uses cell surface receptors and it is internalized via endocytosis. In contrast to LDL, internalization of transferrin appeared to be necessary to promote DNA synthesis (Cuthbert and Lipsky, 1984a). Further studies showed that the inhibitory effect of LDL does not require the binding of LDL to its normal receptors, since the activation of lymphocytes derived from patients with familial hypercholesterolemia (which is associated with abnormal LDL receptors) was also inhibited by LDL (Cuthbert and Lipsky, 1984b). It was also shown that in the absence of endogenous cholesterol synthesis, LDL was able to restore activation of lymphocytes derived from normal donors but did not restore activation of lymphocytes derived from familial hypercholesterolemia patients, suggesting that activation and inhibition of lymphocyte activation are mediated by different receptors. The inhibitory effect of LDL was explained by interference with the stimulatory effect of transferrin (Cuthbert and Lipsky, 1984b).

It was later shown that delipidated ApoE inhibited activation of lymphocytes in the same fashion as LDL (Pepe and Curtiss, 1986). Furthermore, it was shown that ApoE was three times more efficient in suppressing lymphocyte activation than ApoB (another lipoprotein contained in LDL) (Hui et al, 1980). The effect of ApoE in

suppressing lymphocyte activation was shown to be independent of lipoprotein content since delipidated ApoE also suppressed lymphocyte activation (Avila et al, 1982). Interestingly ApoE inhibited early steps of lymphocyte activation such as intercellular accumulation of Ca^{2+} (Hui et al, 1980). ApoE was also shown to inhibit CD4 and CD8+ T lymphocyte proliferation after mitogen activation and this was shown to be associated with a 65% reduction of IL 2 activity. A post-translational modification of IL 2 by ApoE was suggested as the possible mechanism (Kelly et al, 1994).

In summary, early research has demonstrated that lymphocytes have LDL receptors and the coupling of these receptors by ApoE and probably lipoproteins can induce either activation or suppression of the activation and proliferation of lymphocytes after a variety of stimuli.

2.4.2. Studies of ApoE and immunity in animal models

After the development of ApoE deficient animal models, it was shown that ApoE inhibited angiogenesis of AIDS associated Kaposi's sarcoma in a dose dependent manner in ApoE deficient mice. The observed lesions in these mice were smaller and devoid of lymphocyte infiltrate (Browning et al, 1994). *In vitro* studies of cultured Kaposi's sarcoma cells incubated with ApoE, showed that ApoE inhibited DNA synthesis in these cells (Browning et al, 1994). Impaired immunoresponses to *lysteria monocytogenes* in ApoE deficient mice were associated with higher mortality, increased number of colonies and significantly higher levels of TNFα. (Roselaar and Daugherty, 1998). Similarly, levels of TNFα were reported to be 4 to 5 times higher in ApoE knockout mice than in control mice expressing wild type

ApoE (de Bont et al, 1999). These knockout mice were significantly more susceptible to endotoxemia by *klebsiella pneumonie* than control mice (de Bont et al, 1999). Interactions between ApoE and bacterial lipopolisacharide (LPS) was the suggested protective mechanism operating in control mice. More recently, intravenous administration of LPS has been shown to increase plasma levels of ApoE in rodents (Van Oosten et al, 2001). Other studies found that neural glial cultures of ApoE deficient mice when incubated with human ApoE E3 and E4 before stimulation with LPS were associated with decreased accumulation of TNFα in the supernatant (Laskowitz et al, 1997). Conversely, it has been shown that macrophages produce less ApoE after stimulation with certain cytokines and chemokines (Zuckerman et al, 1992). More recent studies expanded these observations and reported inhibition of ApoE secretion by astrocytes in culture mediated by TNFα and INFγ (Starck et al, 2000). *In vitro* activation of microglia (as measured by the production of inducible nitric oxide synthase and IL 1β) by secreted isoforms of amyloid precursor proteins (sAPP) was blocked by ApoE E3 and E4 but ApoE E4 was less effective (Barger and Harmon, 1997). Furthermore, mixed neuronal-glial cultures from ApoE deficient mice produced significantly higher levels of nitric oxide after LPS stimulation than cultures prepared from mice producing wild type ApoE or those expressing human ApoE *ε3* gene (Laskowitz et al, 1998). *In vitro* mRNA expression of TNFα and IL 6 after activation with LPS was greater in microglial cells obtained from ApoE deficient mice than in microglial cells obtained from normal animals. *In vivo*, mRNA expression of TNFα, IL 6 and IL 1β was significantly more up-regulated in ApoE deficient mice than in the control type (Lynch et al, 2001).

Finally, in transgenic ApoE deficient mice suppression of an antiviral cytotoxic T cell response has been shown. In this model, ApoE deficient mice were infected with lymphocytic choriomeningitis virus and antiviral cytotoxic lymphocyte (CTL) responses were measured. A 3 to 5-fold reduction in CTL activity was found in ApoE deficient mice when compared to normal mice. Also, the activation of virus specific CTL cells was significantly impaired in ApoE deficient mice. The hypercholesterolemia observed in these ApoE deficient mice was the suggested cause of the immune impairment observed, although no differences in CTL activation were seen among ApoE deficient mice fed with normal diet and ApoE deficient mice fed with hypercholesterolaemic diet. Normal mice fed with hypercholesterolaemic diet were not susceptible to virus induced immunologic liver disease (Ludewig et al, 2001).

2.4.3. ApoE and Human immunity

Serum of Alzheimer's disease (AD) patients with ApoE 3\4 and 4\4 genotypes produced expression of MHC class II in a significantly greater percentage of microglial cells in culture. The percentage of activated microglial cells incubated in serum derived from AD cases was shown to be lower in *ε2* carriers than in *ε4* carriers. It was also shown that serum derived from AD patients with 3\4 and 4\4 genotypes produced significantly greater release of IL 1β from microglial cells, than serum from AD cases with different genotypes (Lombardi et al, 1998a, 1998b). *In vivo,* significantly increased numbers of activated microglial cells have been reported in brain sections from AD *ε4* carriers than *ε3* carriers, and the total area covered by activated microglial cells (as measured by the expression of MHC class II) was also

significantly greater in *ε4* carriers. Cases with 4\4 genotype had higher numbers and larger covered area than 3\4 cases. Lower numbers of activated microglial cells were observed in AD cases with 3\3 ApoE genotype (Egensperger et al, 1998).

2.4.4. ApoE and viral infections

It has been shown that ApoAI inhibits herpes simplex virus (HSV) induced cell fusion at physiological concentrations by a mechanism involving interactions between viral glycoproteins and the amphipathic helices of ApoAI (Srinivas et al, 1990). The same mechanism has been implicated in the inhibition of HIV-1 infection by ApoAI (Martin et al, 1992; Owens et al, 1990). According to this view the amphipathic helices of ApoAI interact with the hydrophobic domains of the amino-terminus fragment of gp 41, suggesting that this ApoAI exerts its antiviral effects by interfering with the fusion process (Owens et al, 1990; Martin et al, 1992). ApoAI is the main apolipoprotein found in HDL. This lipoprotein mediates the cholesterol efflux from cells in a process that is assisted by ApoAI. The amphipathic α helices of ApoAI promote cholesterol efflux (Reviewed in Fidge, 1999).

Recent studies have shown that ApoE can promote cholesterol efflux from astrocytes and neurons in an isoform specific manner (*ε2* >*ε3* >*ε4*) and the addition of ApoE to astrocytes and neuronal cell cultures is associated with generation of HDL (Michikawa et al, 2000). Interestingly, decreased levels of this lipoprotein (HDL) have been consistently shown in HIV positive patients (Zangerle et al, 1994). Since cholesterol efflux occurs by two main mechanisms, namely, aqueous diffusion and mediated by the amphipathic helices of apolipoproteins, similar actions on

cholesterol efflux between ApoAI and ApoE suggest structural similarities of the amphipathic α helices of these two apolipoproteins. Although no inhibitory actions on HSV cell fusion have been reported for ApoE, recent reports have shown that individuals with certain ApoE genotypes have an increased risk of contracting HSV infections or developing symptoms of infection. An unexpected high frequency of ApoE *ε4* allele (36.3%) was found among herpes labialis sufferers (Itzhaki et al, 1997). The suggested mechanism involved for this increased risk or susceptibility for developing herpes labialis, involved interaction of HSV with HSPG and or LDL receptors in the cell surface (Itzhaki et al, 1998). More recent studies have shown that ApoE *ε2* allele is a risk factor for HSV encephalitis (Lin et al, 2001). The frequency of ApoE *ε2* in 21 cases studied was 26% compared to 7% in 238 normal controls. Again, the suggested mechanism, involved interactions of neurovirulent variants of HSV with a member(s) of the LDL receptor family (Lin et al, 2001).

In summary, it has been shown by a number of experimental studies that ApoE has different actions on the immune system. Actions of ApoE on lymphocytes in culture range from activation to suppression of proliferation and activation. These apparently contradictory actions of ApoE can be explained by activation of intracellular signals after ApoE binding to different receptors of the LDL receptor family. ApoE deficient mice have impaired immunity and these mice have increased susceptibility to endotoxemia and significantly higher levels of cytokines such as TNFα. This cytokine and some chemokines have been shown to increase mRNA expression of ApoE on macrophages and stimulate secretion of ApoE by macrophages. In humans, the effect of ApoE in modulating microglial activation seems to be isoform specific. Apolipoproteins have been shown to interact with viruses especially HSV and HIV-1

and inhibit viral induced cellular fusion or increased susceptibility to viral conditions.

2.5. Effects of ApoE on the CNS

2.5.1. Effects of ApoE on neurite outgrowth

During CNS development and regeneration, a 37 KDa protein, which was later characterized as ApoE has been shown to be present. Early immunohistochemical analysis of optic nerve tracts after optic nerve crushing demonstrated immunoreactivity for ApoE in the degenerating tracts and in their pre-synaptic endings in the lateral geniculate body (Snipes et al 1986).

Levels of ApoE were observed to be elevated in rodent brain by the third day after injury, correlating with the growth cone appearance in injured axons, as well as with the time course of abortive sprouting of the axons (Snipes et al, 1986). Similar results were observed in the Peripheral Nervous System (PNS) after sciatic nerve injury. Subsequent identification of ApoE in the Golgi apparatus in all glial cells in the CNS and non myelinating schwann cells in the PNS supported the association between ApoE metabolism and development and repair of both CNS and PNS (Boyles et al, 1985). Indeed, an isoform specific effect of ApoE on neurite extension and outgrowth was observed in both CNS and PNS (Weisgraber and Mahley, 1996). Cells secreting ApoE E3 showed greater neurite extension than ApoE E4 secreting ones. This action of the ApoE was shown to be dependent on the availability of lipids. Cholesterol rich lipoproteins such as HDL or β-VLDL, are the most efficient lipoproteins in delivering cholesterol to the cells. The most relevant in the CNS is

HDL. It has been established that ApoE E3 has a higher affinity for HDL, while ApoE E4 has affinity for β-VLDL. This difference in lipoprotein avidity for different ApoE isoforms has been linked to the differential effect in remodeling and repair of cells and tissues (Mahley et al, 1998). It is of interest that ApoE/LDL receptor interaction appears to be dependent on the presence of HSPG in the cell surface. Treatment of culture cells with heparinases blocks the lipid uptake by cells even in the presence of ApoE (Mahley et al, 1998). The ability of ApoE to stimulate neurite outgrowth and extension involved in the repair mechanism has been linked to the ApoE action on retrograde transport of cholesterol from the cell. This efflux of cholesterol in the CNS appears to be isoform specific, with ApoE E2 being more efficient as lipid acceptor than the other ApoE isoforms.

The lipoprotein involved in the cholesterol uptake has a density similar to the HDL (1.07 and 1.21 g/ml)(Michikawa et al, 2000). Protein kinase C and lactoferrin inhibitors are able to significantly reduce the cholesterol efflux from the cells. Lactoferrin acts as a selective inhibitor of the HSPG/LRP pathway, suggesting similar mechanisms for cholesterol efflux and uptake. This was observed in cultured rat neurons and astrocytes which suggests that the efflux of cholesterol from astrocytes and neurons use the same pathway (Michikawa et al, 2000).

Neurite outgrowth can also be inhibited by depolymerisation of microtubules. This has been observed in neuronal cell cultures. In that system, the addition of ApoE E4 plus a source of lipids mediated by β-VLDL type lipoproteins resulted in a less polymerised tubulin with reduction of neurite outgrowth. The exchange of ApoE E4 for E3 in the culture media resulted in extensive formation of microtubules, which were morphologically intact.

As mentioned above, the effect of ApoE on neurite outgrowth could be mediated by HSPG/LRP pathway. The first step of this process involves binding of ApoE containing lipoproteins with the cell surface proteoglycans (HSPG). After this initial binding, ApoE is transferred to the LRP receptor and internalized or alternatively the HSPG/LRP complex is internalized in total. This is the most likely mechanism involved in the isoform specific action of ApoE on neurite outgrowth. For example, ApoE E2 has a limited or defective ability to bind to LDL receptor, but ApoE E2-containing β-VLDL, has a stimulating effect on neurite outgrowth because of the ability to bind HSPG/LRP complexes. This HSPG/LRP complex is also required for ApoE E4 containing lipoproteins to exert maximal neurite outgrowth inhibition.

In the PNS, the type of lipoprotein supplied to sympathetic neurons modulates the axonal growth in specific manner. In this case, LDL is a more efficient cholesterol provider than cholesterol-containing HDL. It is important to highlight that LDL receptors are present in axons, while LRP receptors are found predominantly in neuronal cell bodies, suggesting that the lipoprotein effect on axonal growth is also modulated by the distribution and type of lipoprotein receptors in a particular anatomical area (Posse de Chaves et al, 1997).

In the CNS, transgenic mice expressing human ApoE E3 and E4 have a differential effect on neurite outgrowth of hippocampal neurons, with ApoE E3 being more effective in promoting neurite outgrowth than ApoE E4. This effect appeared to be related to the expression of LRP rather than to the level of ApoE, since ApoE levels were similar in both transgenic mice lines. This stimulating effect of ApoE E3 was blocked in cultured neurons derived from these mice by the addition of LRP receptor associated protein (RAP) which is an antagonist of all LRP ligands (Sun et al, 1998).

It should be noted that in that *in vitro* model, LRP were found in all neurite processes, while in more mature cells they are restricted to the somato-dendritic domain of hippocampal neurons. (Sun et al, 1998).

2.5.2. Effect on neurodegeneration

Apolipoprotein E (ApoE) is the major apolipoprotein found in the CNS. Other apolipoproteins recovered from the CSF include ApoAI and ApoJ.

Namba (Namba et al, 1991) and co-workers, studying lipid distribution in the CNS of Alzheimer's disease (AD) patients, unexpectedly found immunohistochemical reactivity with monoclonal and polyclonal antibodies against human ApoE in senile plaques, blood vessel walls, and neurofibrillary tangles. This ApoE immunoreactivity was also found in amyloid plaques in cases of Creutzfeldt-Jakob disease (CJD) and in the amyloid deposits in the blood vessel walls of cases of amyloid angiopathy. This was the first link between ApoE and neurodegenerative conditions and these results suggested a close association between ApoE and amyloid β (Aβ) peptide.

The most conclusive evidence linking ApoE with AD is provided by the ApoE genotype of familial and sporadic late onset AD. Over-representation of ApoE *ε4* allele in a heterogeneous group of late onset familial AD cases was found in 1993 (Strittmatter et al, 1993). Soon after, these results were confirmed, and the observations were extended to sporadic AD cases (Saunders et al, 1993). It was later shown as well, that the risk of developing AD was gene dose dependent and this risk increased by a factor of 2.84 for each additional ApoE *ε4* allele (Corder et al, 1993). Age of onset of the disease was also observed to relate to the ApoE genotype. Thus, patients with one ApoE *ε4* allele had an average age of onset of the disease of 68.4

years in contrast to 75.5 years for patients without an ApoE *ε4* allele (Corder et al, 1993; Poirier et al, 1993; Rebeck et al, 1993). The over-representation of the ApoE *ε4* allele has been reported in late onset sporadic AD cases in many different ethnic populations, even in Japan where the frequency of ApoE *ε4* is one of the lowest in the world. Although, there are important differences in the ApoE genotype among different populations, in most of them an increased frequency of ApoE *ε4* allele is observed in AD cases (Mayeux et al, 1993; Brousseau et al, 1994; Peacock et al, 1994; Kalman et al, 1997). A notable exception is the African-american population. In this group, there is an overall high frequency of the *ε4* allele and no association between ApoE *ε4* and AD has been found (Mayeux et al, 1993).

It is currently accepted that the ApoE *ε4* allele is a risk factor for developing late onset AD, while ApoE *ε2* allele is regarded as a protective factor against sporadic AD and other dementing illnesses such as sporadic CJD (Corder et al, 1994; Pickering-Brown et al, 1995).

2.5.2.1 ApoE and β amyloid

Initial observations regarding the high affinity of ApoE in binding Aβ *in vitro* (Strittmatter et al, 1993), and the isoform specific interactions between ApoE and Aβ (ApoE E4 being more effective in the formation of ApoE/Aβ complex), suggested that this was the mechanism involved for the increased risk of development of AD for *ε4* carriers. These ApoE/Aβ complexes give rise to precipitation of Aβ monofibers. These studies linking ApoE binding to Aβ peptide *in vivo* and *in vitro* were based on the Aβ that was deposited in the neuropil. This can be explained

because ApoE synthesis in the CNS had been demonstrated in astrocytes and glial cells, which were thought to be the major and almost exclusive source of ApoE in the CNS since ApoE production from macrophages in the normal CNS is limited (Boyles et al, 1985). More recent studies have shown that neurons can produce ApoE and ApoE mRNA has been demonstrated in frontal and hippocampal neurons. This neuronal ApoE mRNA synthesis seems to be restricted to certain neuronal sub-populations, since ApoE mRNA synthesis has not been observed in cerebellar purkinje cells (Xu et al, 1999). It is interesting that neuronal ApoE mRNA correlates to brain areas rich in Aβ deposits in AD cases. Recent studies showed that ApoE/Aβ association is an event tightly linked to the secretion of amyloid precursor protein (APP). *In vitro* studies demonstrated an ApoE isoform specific effect in APP secretion, in that ApoE E4 is the isoform associated with the highest level of secreted APP (sAPP). This effect on APP secretion seems to be mediated by cytoplasmic retention of APP with the other two ApoE isoforms (Ha et al, 1998). A different *in vitro* model using smooth muscle cells confirmed the secretion of the whole APP molecule by ApoE E3 and E4 stimulated cells, and the intracellular accumulation of the secreted forms of Aβ (40 amino acids long or Aβ40). This was associated with a decrease culture cell density and reduced viability of the smooth muscle cells, suggesting a direct effect of Aβ in cell degeneration (Mazur-Kolecka et al, 1999). In the human brain immunocytochemical co-expression of ApoE and Aβ in the neuronal cytoplasm in AD cases has been observed. This co-expression of ApoE/Aβ colocalized with DNA damage in susceptible neuronal populations as evidenced by terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) (LaFerla et al, 1997).

The immunohistochemical identification LRP (a receptor for both ApoE and APP), in senile plaques in AD cases, neuronal cell bodies of granular cells of the dentate fascia and pyramidal neurons of the CA4, CA3 and CA1 sectors of the hippocampus, offers a different explanation for the intracellular co-localization of ApoE/Aβ (Rebeck et al, 1993). According to these findings, ApoE mediates the endocytosis of Aβ via LRP. This is meant to be followed by endosomal/lysosomal degradation of Aβ, suggesting a catabolic fate for Aβ in the endosomal/lysosomal compartment. This observation is not completely certain because only a small proportion of the secreted Aβ is again internalized via endocytosis (for review see Wilson et al, 1999, Storey and Cappai, 1999). However the exact mechanism of ApoE/Aβ interaction in AD remains obscure.

2.5.2.2. ApoE and neurofibrillary tangles

The other histological hallmark of AD, is the neurofibrillary tangles. The presence of ApoE immunoreactivity in neurofibrillary tangles has been demonstrated in both intracellular and extracellular neurofibrillary tangles (Namba et al, 1991; Yamaguchi et al, 1994). This finding focused the attention towards ApoE and the microtubule associated protein tau. This interaction also seems to be ApoE isoform specific. The formation of stable complexes between ApoE E3 and tau contrasts with the poor ability of ApoE E4 to form complexes with tau (Weisgraber and Mahley, 1996). This ApoE tau interaction seems to be dependent on the phosphorylation state of tau, since hyperphosphorylated tau does not form complexes with ApoE E3. In the AD context ApoE E3 appears to inhibit the phosphorylation of tau delaying in that way the formation of paired helical filaments (PHF) characteristic of the neurofibrillary

tangles. It has been hypothesized that ApoE E4 binds tau, altering tau susceptibility to kinases and phosphatases, which are the enzymes responsible for tau phosphorylation, therefore, favoring hyperphosphorilation of tau. Carriers of ApoE *ε4* allele have higher tau levels in the CSF. This is still a controversial matter, specially because phosphorylation independent interaction between tau and sulphated glycosaminoglycans (Heparin and Heparan sulphate) lead to the formation of tau filaments in cells, independent of any ApoE interaction (Billingsley and Kincaid, 1997; Johnson and Hartigan, 1998; Tonlay and Probst, 1999). Further evidence concerning the role of sulphated glycosaminoglycans and PHF formation, include observations of increased phosphorylation of tau, stimulated by glycosaminoglycans. Heparan sulphate immunoreactivity co-localized with the so-called pretangle neurons, suggesting that the accumulation of heperan sulphate glycosaminoglycans precedes tau hyperphosphorylation (Tonlay and Probst, 1999). It is interesting that the pretangle stage correlates with DNA damage illustrated by TUNEL positivity (Sheng et al, 1998).

In other neurodegenerative disorders such as Parkinson's disease, the frequency of the *ε4* allele of ApoE has been controversial. The French Parkinson's disease genetics study group (the FPDGSG, 1997) reported a similar frequency of ApoE *ε4* allele between Parkinson's disease patients and controls, but reported an over-representation of the *ε2* allele among familial Parkinson's disease patients, compared with controls, although the difference did not reach statistical significance. In contrast, recent studies have shown a significantly increased frequency of ApoE *ε4* allele among early onset Parkinson's disease (P=0.03) (Kruger et al, 1999). When comparing Parkinson's disease patients and controls for the combination of alleles for

α-synuclein and ApoE, the genotype combination α-synuclein allele 1 and ApoE *ε4* allele was more common in Parkinson's disease patients, particularly in those with early onset (P=0.005) (Kruger et al, 1999). The average age of onset in sporadic Parkinson's disease patients was also influenced by the ApoE genotype in an isoform specific manner. In Parkinson's disease cases, patients homozygous for the *ε4* allele had an average age of onset of 45.25 years compared to 56.97 years in patients without an ApoE *ε4* allele (Kruger et al, 1999). Furthermore, ApoE *ε4* carriers have a greater number of cortical Lewy bodies. The frequency of the *ε4* allele among patients with Parkinson's disease and AD type pathology was found to be higher (29.4%) than for cases without those histological findings (Mattila et al 1998).

In Multiple Sclerosis (MS), contradictory results have been published regarding the association of ApoE and the course of the disease. The ApoE *ε4* was reported more common in more aggressive cases of MS (Evangelou et al, 1999). It has been claimed that *ε4* carriers progress in their disability (Chapman et al, 1999) suggesting an impaired recovery after relapsing episodes of the disease. Other studies however have not found associations between ApoE *ε4* allele and clinical progression of MS (Weatherby et al, 2000). Recently, it has been suggested that remyelination in MS patients with ApoE *ε2* allele may be defective since no histological evidence of remyelination in 7 MS cases with this allele was seen, but, it was observed in 39% of cases without the *ε2* allele (Carlin et al, 2000).

In argyrophilic grain disease (a disease characterized by abnormally phosphorylated tau and cytoskeletal changes in oligodendrocytes and neurons) a high frequency of ApoE *ε2* allele (22%) was found in 48 patients, while the frequency of the same allele in controls was 4% (Ghebremedhin et al, 1998).

2.5.3. ApoE and head injury

The effect of ApoE *ε4* allele is not restricted to neurodegenerative dementing illnesses. It has also been observed that the frequency of *ε4* carriers in head injured patients with Aβ deposits is higher (52%) than in head injury subjects without Aβ deposits in the brain (16%) (Nicoll et al, 1995; Horsburgh et al, 2000). Indeed Aβ deposition following head injury is a well-known event presenting shortly after the injury (Roberts et al, 1990). A poorer outcome after head injury has also been reported in *ε4* carrying individuals (for review see Graham et al, 1999). Furthermore the frequency of ApoE *ε4* among patients who did not recover from post-traumatic coma was found higher (42%) compared with patients who recovered consciousness (Sorbi et al, 1995). Excitotoxicity has been implicated in head trauma as an important injury mediator and it appears that ApoE can also modulate the brain response to excitotoxic insults. After excitotoxic insults, ApoE deficient mice have a significant loss of synaptophysin in presynaptic terminals compared with their littermate transgenic mice expressing human ApoE E3. Expression of human ApoE E3 protected those mice against excitotoxic damage while ApoE E4 expression had a very limited effect against excitotoxicity (Buttini et al, 1999). In this same model, human ApoE E3 expression was shown to be protective against the age-related neurodegeneration observed in ApoE deficient mice (Buttini et al, 1999). The outcome of CNS ischaemic insults has also been shown to be modulated by ApoE in an isoform specific manner. Transgenic mice expressing either the human ApoE *ε3* or *ε4* gene had their middle cerebral artery occluded for up to 60 minutes and the size of the infarcts in the cortex and subcortical areas were measured. Significant differences in infarct size were found among *ε3* and *ε4* expressing mice. Transgenic

mice expressing *ε4* had larger infarct areas in both cortex and subcortical areas (Sheng et al, 1998). The mechanism suggested for the above observation included a neuroprotective role of ApoE against oxidative stress and/or the effect of ApoE in down-regulating the inflammatory response of the CNS (Sheng et al, 1998). Supporting the first view, it was shown that ApoE deficient mice have defective antioxidant mechanisms in their brain (Lomnitzki et al, 1997). Recent studies have also shown that ApoE, in an isoform specific manner, binds 4-hydroxynonenal, which is produced by oxidation of the fatty acids of cell membranes and has cytotoxic effects by impairing the functions of glutamate transporter protein in astrocytes. The ApoE E2 protein binds larger amounts of this compound (4-hydroxynonenal) than ApoE E4 protein, which is the isoform with least binding activity.

2.5.4. ApoE and cerebral amyloid angiopathy

In cerebral amyloid angiopathy (CAA), a high frequency of ApoE *ε2* carriers has been reported (Nicoll et al, 1997) and it has been claimed that the possession of an *ε2* allele of ApoE increased the risk of heamorrhage in CAA (Nicoll et al, 1997). Similar results were reported in a histological study of CAA haemorrhage in which 33% of the haemorrhage cases due to CAA had an *ε2* allele (McCarron et al, 1999). These findings have not been confirmed by others (Garcia et al, 1999).

2.5.5. ApoE and HIV associated dementia

In the setting of HIV infection, the role of ApoE has not been studied in detail. There are few and contradictory reports concerning the role of ApoE and specifically the

presence of the *ε4* allele in HIV associated dementia. (HAD). This condition is characterized by a cognitive decline, usually accompanied by behavioral changes and motor slowing (for review see Clifford, 2000; Bell et al, 1998). An excess of dementia and peripheral neuropathy has been reported in HIV positive individuals who also had an ApoE *ε4* allele compared with other HIV positive individuals who did not have an ApoE *ε4* allele (Corder et al, 1998). In this report, there was a significant association between having an *ε4* allele and clinical consultations because of neurological symptoms ($p<0.0001$) (Corder et al, 1998). In contrast, no association between the *ε4* allele of ApoE and HAD was reported in another study (Dunlop et al, 1997).

Chapter 3

DRUG USE

3.1 Overview

Intravenous drug use is a recognized risk factor for contracting blood borne infections including HIV infection. Heroin introduced in 1898 as a substitute of morphine for clinical use (Van Ree et al, 1999), has been the main drug of abuse among HIV positive cases. Heroin is a derivative of opium, which is extracted from the seed capsule of *Papaver Somniferum* (poppy). The actions of opium have long been studied and the term opioid is applied to substances with an opiate-agonist action (for review seeVan Ree et al, 1999). Early studies demonstrated that opiates mediated their action by stimulation of different type of receptors in the CNS. Three different types of opioid receptors are recognized, namely, μ (mu), κ (kappa) and δ (delta), which are also known as MOR, KOR and DOR respectively. These receptors have also been identified in cells of the immune system including T and B lymphocytes and macrophages (Carr et al, 1989; Bidlack et al, 1992; Chuang et al, 1994; Gaveriaux et al, 1995; Sedqi et al, 1995). The role of these receptors in the immune system varies widely.

3.2 Effects of opiates on cells of the immune system

Animal models have shown that chronic administration of morphine reduced T and B lymphocyte proliferation in response to mitogens. This effect is modulated by the doses of morphine used since small doses were shown to have the opposite effect (Bryant et al, 1987, 1988). More recent studies demonstrated that proliferation of activated T lymphocytes is suppressed by heroin use. The total number of CD4 cells was found to be significantly decreased in heroin addicts and conversely the total number and the percentage of cytotoxic CD8 T cells and B lymphocytes were found significantly increased (Govitrapong et al, 1998). Reduced numbers of NK cells in humans and diminished cytolytic activity of these cells *in vitro* have also been documented. The effect on NK cells activity was shown to be mediated by opioid receptor activation since the effect of morphine administration was reversed by opioid receptor antagonists (Bayer et al, 1990; Bhargava et al, 1994; Govitrapong et al, 1998).

Opiates also decrease INFγ production in human peripheral blood mononuclear cells (PBMC) and in mice by a mechanism involving opioid receptors (Peterson et al, 1987; Lorenzo et al, 1987). Human cultured monocytes appeared as specific targets for opioid induced reduction in INFγ production (Peterson et al, 1987). Impairment in macrophage response to chemokines associated with chronic morphine use has also been documented *in vitro*. A 65% decrease in the response to macrophage colony stimulating factor (M-CSF) by mice bone marrow cells has been observed. In the same model, no abnormalities in bone marrow cell response to granulocyte/macrophage colony stimulating factor (GM-CSF) were observed. Again

the opioid receptor antagonist, Naloxane, blocked the inhibitory effect of chronic morphine administration (Roy et al, 1991). Reduced production of IL 2 and IL 4 in mice associated with chronic morphine administration have also been shown but no significant effect on induction of cytotoxic T cells was observed in these mice. In contrast, a significant decrease in induction of these T cells was observed in abstinent mice (Bhargava et al, 1994).

Morphine administration has been shown not to affect basal production of TNFα by PBMC *in vitro*. The production of this cytokine by PBMC after stimulation with lipopolysaccharide (LPS) was also shown to be unaffected in the same model (Chao et al, 1992). Non-effect on basal TNFα production have also been observed *in vivo* (Bhargava et al, 1994). Other *in vitro* studies have reported inhibition of TNFα release by PBMC after stimulation with phytohemagglutinin, when cells were incubated for 24 hours with morphine prior to stimulation (Chao et al, 1993). Morphine has been shown to stimulate the production of transforming growth factor beta (TGFβ) by PBMC *in vitro* after stimulation with LPS (Chao et al, 1992), and TGFβ appears to counteract the effect of morphine on TNFα production by PBMC *in vitro* (Chao et al, 1993). Cultured PBMC derived from normal human donors showed increased production and mRNA expression of MCP-1, IP 10, and RANTES when incubated with MOR agonists. These findings suggest that MOR agonists act at the transcriptional level (Wetzel et al, 2000).

3.2.1. Effects of opiates on microglial cells

Morphine incubation of murine microglial cell cultures produced significantly larger amounts of TNFα after stimulation with LPS. This treatment was associated with a significant increase in superoxide anion production by these cells (Chao et al, 1994). Recent *in vitro* studies have shown that naloxane prevents activation of microglial cells in culture and the subsequent release of cytokines such as TNFα and IL1β and nitric oxide derivatives including peroxinitrite by these cells. Interference of naloxane with LPS binding to its receptor on microglial cells is the suggested mechanism for these actions (Liu et al, 2000).

3.2.2. Effects of opiates in HIV infection

After infection of PBMC with T-tropic HIV-1, cells which were previously incubated with MOR agonists showed increased basal production of regulated upon activation normal T cell expressed (RANTES) and inflammatory protein (IP 10), but this effect was not observed after activation of infected PBMC with phytohemagglutinin (Wetzel et al, 2000). An opposite effect was observed in PBMC infected with M-tropic HIV-1. In this model, pretreatment of PBMC with MOR agonist before infection with M-tropic HIV-1 resulted in 13 fold increase in RANTES production after activation with phytohemagglutinin (Wetzel et al, 2000). It has been shown that CD4+ T cells express DOR and KOR (Sharp et al, 2001). Incubation of HIV infected CD4+ T cells with DOR and KOR agonists before stimulation of these cells with phytohemagglutinin resulted in inhibition of HIV-1 p24 antigens expression by these cells (Sharp et al, 2001; Peterson et al, 2001). In cultured microglial cells, KOR agonists also suppressed HIV-1 antigen expression

(Chao et al, 1997). The mechanism proposed for the inhibition of HIV antigen expression by CD4+ T cells and microglial cells, involved transdeactivation of chemokine receptors by opioid receptor agonists (Sharp et al, 2001, Peterson et al, 2001). All of these opioid receptors (such as chemokine receptors) are seven transmembrane spanning receptors, which are associated with G proteins (Li et al, 1993; Chen et al, 1993). When agonists bind these receptors, phosphorylation of the receptor by G protein-coupled kinases can result in homologous desensitization and internalization of the receptor. Heterologous desensitization (transdeactivation) consists of loss of receptor function after phosphorylation of different receptors (Grimm et al, 1998; Wang and Oppenheim, 1999).

In summary, opioid receptor stimulation can exert a number of effects on the immune system. Most of the studies have shown different deleterious actions of morphine administration in various immune cell types. Most of the studies were carried out in cultured cells or in rodent animal models. Some pharmacological studies have established certain anti-HIV infection effects of opioid receptor agonists. In humans studies are needed to validate these results. However, it has been shown that the number of CD4+ T cells is decreased in HIV negative heroin addicts while the total number and percentage of CD8+ T cells is increased suggesting that heroin has a differential effect on different T cell populations (Govitrapong et al, 1998).

Neuropathological studies in HIV negative drug users have shown activation of microglial cells and astrocytosis in the hippocampus, suggesting a direct effect of the drugs of abuse on microglial activation (Oehmichen et al, 1996). In Edinburgh, an

increased number of CD8+ T lymphocytes and activated microglial cells in brain sections of HIV positive drug users compared with HIV negative drug users has been reported (Tomlinson 1999). In HIV negative drug users a significantly greater number of activated microglial cells were observed compared to non-drug users controls (Tomlinson et al, 1999). Interestingly, in this study, no significant differences in the number of activated microglial cells between HIV positive and HIV negative drug users was found. In this same cohort of cases, the frequency of HIVE among drug users was shown to be significantly higher than in MSM cases (Bell et al, 1996, 1998).

Chapter 4

CASES AND METHODS

4.1. Study Population

All studies were approved for research by the Lothian Ethics of Research Committee. Studies on live patients Groups I and II were separately approved in addition.

The study population for each experiment is described separately.

4.1.1. ApoE genotyping

The ApoE genotyping was carried out in five different groups. Groups I and II consisted of alive normal DNA donors and HIV negative drug users. In these cases DNA was extracted from buccal smears. The selection of cases of group I and II was based in the voluntary acceptance to take part in the study. Cases were anonymized to comply with the ethical regulations of the University of Edinburgh.

Groups III to V were extracted from the HIV Neurobank of the Department of Neuropathology of the University of Edinburgh. The case selection was based in the availability of frozen tissue. The cause of death of these cases is displayed in appendix II. No tissue was available for pre-symptomatic HIV positive non-drug user cases. Cases were selected and processed blind to age, gender, risk factor to contract HIV or cognitive status. Once the ApoE genotyping was completed, cases were divided in 5 main groups:

Group I consisted of 64 normal control cases (alive).

Group II consisted of 82 HIV negative drug users (alive).

Group III consisted of 38 pre-symptomatic HIV positive drug users (dead).

Group IV consisted of 84 HIV positive drug users with AIDS (dead).

Group V consisted of 44 non-drug users with AIDS of which 6 were haemophiliacs and 38 were men who have sex with men (MSM) (dead).

Thus the total number of cases used for ApoE genotype comparisons among risk factor groups was 312. Published data for ApoE genotype for a cohort of 400 normal adults from Grampian, Scotland (Cumming and Robertson 1984) was used for comparisons and these cases were analysed as Group VI.

When comparing the frequency of ApoE genotypes and alleles among risk factor groups III and IV were analyzed together adding up to a total of 122 cases.

Besides the analysis concerning ApoE genotype, differences between the study population groups was also assessed regarding CD4/CD8+ T cell counts, histological examination of the brain and CD68 positivity in different brain areas.

4.1.2. CD4/CD8+ T cell counts

For analysis of CD4/CD8 T cell counts, only cases with less than 200 CD4+ T cells or AIDS defining illnesses were used. This allowed comparisons between cases at the same stage of HIV disease. All of these cases were in Groups IV and V. Eight cases with AIDS defining illnesses, which either had CD4 counts above 200 or which had no record for CD4 counts were excluded. These cases were 6 in Group IV and two in Group V. Four cases in Group IV had no CD8+ T cell counts available for this study. The total number of cases included was 120 of which 78 were in Group IV and 42 in Group V.

4.1.3. Histological examination of the brain.

Detailed histological examination of the brain was carried out in 78 cases, 49 were in Group IV and 29 in Group V. Cases were selected from the Edinburgh HIV Neurobank file in chronological order blind to group and ApoE genotype.

4.1.4. CD68 Quantitation

Analysis of CD68 quantitation was carried out on 36 cases. Four cases were in Group I, 10 in Group II, 7 in Group III, 9 in Group IV and 6 cases were in Group V. All the cases in Groups IV and V had HIVE. Cases were analyzed blind to group and ApoE genotype.

4.2 Clinical data

Relevant clinical information from selected cases was obtained from the data bank of the Department of Neuropathology of the University of Edinburgh. Additional information about the cognitive status of selected cases was obtained, when available, from the Infectious Disease Department file of the Western General Hospital, Edinburgh.

4.3 Cases for histological examination

Histological examination was carried out in unselected cases. All cases genotyped for ApoE were listed and histological examination of all sections available for each case was carried out in numerical order blind to age, gender, cognitive status and risk factor. Autopsy samples used for the histological examination were obtained from

the HIV neurobank of the department of pathology of the University of Edinburgh, Western General Hospital, Edinburgh. Cases selected for the study were assessed by post-mortem examination and DNA extraction

4.3.1. Post-mortem Examination

All post-mortem examinations were carried out within 3 days of death. At the time of the post-mortem examination, tissue samples form different organs including brain, were frozen and stored at –80°C. Frozen brain tissue was used for DNA extraction for ApoE genotyping.

The brain was fixed in 10% formalin for three weeks. The macroscopic examination and tissue sampling followed this. Sections from frontal, parietal parasagital, central white matter, occipital lobe including the visual cortex, midbrain, basal ganglia, thalamus, temporal lobe including the hippocampal formation and entorhinal cortex, medulla, cerebellum and spinal cord, among others were taken for histological examination. Selected tissue was dehydrated and paraffin-embedded.

4.4. Histological Sections

Histological sections were used for immunohistochemical demonstration of CD68 antigen.

Paraffin-embedded tissue sections of 3-5μm were obtained using Reicher-Jung biocut 2030 rotary microtome. Sections were mounted on superfrost plus glass slides (BDH laboratories companies) or on 0.1% poly-L-Lysine (Sigma) coated slides. Overnight incubation at 37°C was allowed before immunostaining.

4.4.1. Immunostaining

Procedure

4.4.1.1. Re-hydration

Sections were placed in two xylene (Genta Medical) baths for 5 minutes each, to remove wax. A sequential passing of 5 minutes each through industrial methylated spirits (Genta Medical) and 70% alcohol was required for re-hydration. This was followed by 15-minutes bath in saturated alcoholic picric acid solution to clear the formalin present in tissue sections. Tap water wash of 15 minutes removed the picric acid excess on tissue sections.

4.4.1.2. Antigen Retrieval

Antigen retrieval is a procedure routinely applied to formalin-fixed tissue before immunostaining. Formalin fixation of fresh tissue induces the formation of protein cross-linking, limiting the antigen identification on paraffin- embedded tissue.

Techniques based on high temperature, proteolytic enzymes and detergent treatment are commonly used for antigen retrieval. The use of a specific antigen retrieval procedure depends on the antigen to be tested. In the present study, proteolytic enzyme digestion was used. This technique is routinely applied and has been optimized in the department of Neuropathology of the University of Edinburgh, at the Western General Hospital.

4.4.1.2.1. Proteolytic Enzyme Digestion

<u>Reagents</u>

0.1% Trypsin (ICN Flow Laboratories) (final concentration)

0.1% Calcium Chloride (Sigma) (final concentration)

0.0125M Tris-buffered saline (TBS) Ph 7.8 (final concentration).

Procedure

Approximately 400 ml. Solution containing the above reagents were placed in preheated bath at 37°C for 30 minutes. When the temperature of the solution reached 37°C, sections were placed in and incubated for 30 minutes. This was followed by a 5-minute rinse on tap water in order to stop the proteolytic process.

4.4.1.3. Blocking of endogenous peroxidase

Blocking of endogenous peroxidase was carried out by ten-minute bath in 3% solution of hydrogen peroxide (Fisher Scientific International Company) in distilled water. A 5-minute bath in water removed the hydrogen peroxide.

4.4.1.4. Blocking endogenous biotin

Biotin is present in some human tissues including brain. Due to the marked affinity of avidin for this protein, immunodetection systems based on avidin and biotinylated horseradish peroxidase (HRP) can be potentially masked by non-specific background staining. Some methods have been used to reduce background staining, including blocking of endogenous biotin. The use of 5% fat-free milk powder can block endogenous biotin but the results are not always consistent. In the Department of Neuropathology of the University of Edinburgh, the systematic use of commercial preparations is preferred.

Reagent

Avidin-biotin blocking kit (Vector Laboratories sp-2001) consists of aviding blocking reagent and biotin blocking reagent. After blocking with normal serum, the

avidin-blocking reagent is applied for 15 minutes, then a rinse with washing buffer is followed by 15 minutes incubation with biotin blocking reagent. This blocking procedure must be done before the incubation with the primary antibody.

4.5 Immunostaining techniques

Standard avidin-biotin complex technique was used for immunostaining with CD68 (PG-M1, DAKO). This method has been optimized in the Neuropathology Department of the University of Edinburgh at the Western General Hospital.

Reagents

3% H_2O_2 in distilled water

Proteolytic antigen retrieval solution (above)

Tris-Buffered Saline (TBS): 0.05m Tris/HCL, 0.15M NaCl Ph 7.6

Normal rabbit serum 1:200, diluted in TBS 0.1% bovine serum albumin (BSA)

Bovine Serum Albumin (BSA)

Avidin-biotin blocking kit (Vector Laboratories)

Monoclonal CD68 (PG-M1, DAKO), 1:200 diluted in TBS (primary antibody)

Biotinylated rabbit-ant-mouse secondary antibody (DAKO) 1:200 diluted in TBS (secondary antibody)

StreptAB complex/HRP (DAKO)

Diaminobenzidine (DAB)

Meyer's haematoxilin

Mounting medium (DPX)

Procedure

Sections were re-hydrated as mentioned; the endogenous peroxidase was blocked by 10 minutes incubation in 3% H_2O_2. Sections were rinsed in tap water for 5 minutes. Incubation for 30 minutes in 0.1% trypsin and calcium chloride at 37°C as described, was followed by 5-minute rinse in tap water. Incubation at room temperature in TBS for 15 minutes was followed by 30 minute incubation in normal rabbit serum diluted in TBS plus 0.1% BSA. Blocking of avidin for 15 minutes followed by a 5-minute rinse with TBS and Biotin blocking for 15 minutes followed this. Five minute rinse with TBS preceded the addition of the primary antibody (CD68) 1:200 diluted in TBS plus 0.1% BSA. The incubation time for the primary antibody was 30 minutes. The secondary antibody was added after two rinses in TBS of 5 minutes each. The incubation time of this secondary antibody was 30 minutes. Rinsing the sections twice in TBS for 15 minutes each and the addition of the streptAB Complex/HRP followed this. After 30 minutes incubation, sections were rinsed in TBS twice and the reaction was visualized using DAB. Sections were then placed in tap water, counter stained with Meyer's haematoxylin, cleared by sequential rinses in increasing concentrations of alcohol and xylene and mounted with mounting medium (DPX). Sections were ready for light microscope examination.

4.6 DNA extraction

Specific safety measures were taken when manipulating the tissues. These included wearing coats appropriate to those procedures, plastic apron, disposable cuffs and use of a class 1 microbiological safety cabinet when exposing frozen tissue or fresh fluids from HIV-1 infected patients.

Adequate disposal of all the contaminated items, after disinfecting was ensured as well as decontamination of all surfaces

4.6.1. DNA extraction from frozen tissue

Reagents:

TNE buffer

Reagent	Final Concentration
4M NaCl	0.11M
1M Tris Ph 8.0	55mM
0.2M EDTA Ph 8.0	1.1M
10% Sodium-*n*-Laurylsarcosine (SDS)	0.55%

Other Reagents

Phenol H_20 saturated (Sigma)
Chloroform: ISO-Amyl Alcohol (AnalaR)
50:1 (v/v)
3M Na acetate Ph 5.2
80% Ethanol
Poly A (DNA carrier) 40 μg/ml
Proteinase K (Sigma) 100μg/ml
Lysis buffer: TNE buffer as above, 100μg/ml proteinase K, 40μg/ml poly A.

Procedure

Frozen tissue was obtained from the HIV Neurobank of the University of Edinburgh, Neuropathology Department. DNA extraction from frozen tissue from HIV infected cases and HIV negative drug users, was carried out in containment level 3 facility in a class 1 safety cabinet since such material is considered a biological hazard "group

3". Chain mail gloves between rubber disposable gloves were worn for cutting frozen tissue. Frozen tissues were dissected and a 0.5cm sample from each case was obtained and placed into a previously marked clean 1.5 ml Eppendorf tube, containing 500µml of lysis buffer. Samples were vortexed and incubated at 65°C for a variable period of time, no shorter than 2 hours. After lysis, 450µl of phenol was added to the Eppendorf and the content was thoroughly mixed by vortexing for 5 minutes. Centrifugation at 13000 rpm at room temperature for 10 minutes separated the DNA containing aqueous layer from other proteins, which were precipitated. The aqueous (upper layer) was transferred to a clean Eppendorf and the phenol purification step was repeated as above. The upper aqueous, DNA containing layer was transferred again to an eppendorf tube containing 450µl of a solution of 50:1 (v/v) of chloroform/iso-amyl alcohol (AnalaR). A two-minute vortexing preceded a 10 minute centrifugation at 13000 rpm at 4°C for 10 minutes. This step aimed to further precipitate proteins leaving the DNA-containing aqueous layer ready to be transferred. The aqueous layer was transferred to an eppendorf tube containing 800µl of 100% ethalnol (AnalaR) and 40µl of 3M Na acetate Ph 5.2. Contents were mixed by inverting 25-50 times and left overnight at –20°C or 1 hour at –40°C. The latter step precipitated the DNA. The lysate was centrifugated at 10000 rpm at 0°C for 30 minutes. The supernatant was discharged and the pellet (DNA) was washed in 600µl of 80% ethanol followed by a 5 minute centrifugation at 10000 rpm at 0°C. The supernatant was discharged, and the pellet was dried at room temperature or at 42°C for 10 minutes before re-suspending it again in 25µl of double distilled water. The extracted DNA was left at room temperature overnight. The DNA was stored at –80 for later use.

4.6.2. DNA extraction from buccal smears

For the extraction of DNA from buccal smears a DNA isolation kit D-3300A (Gentra Systems), was used. Buccal smears were stored at –20ºC upon arrival to the Neuropathology Department of the Western General Hospital. A code name for each case was produced stating the origin of the sample, the risk factor and the age of the patient. Normal DNA donors had different code names.

Every 3 weeks cases were processed in a containment level 3 facility, using a class 1 safety cabinet.

The cytology sample was collected in a nylon bristle cytology brush, which was sent sterile to the clinics participating in the collection of samples.

The DNA isolation kit consisted of:

Lysis buffer, containing anionic detergent and an inhibitor of DNAases.

RNAase enzyme

Protein precipitation solution

Glycogen solution (20 mg/ml)

Other reagents:

100% isopropanol

70% ethanol

Proteinase K 20mg/ml (final concentration 10μg/μl)

Procedure

The brush was dipped several times in a 1.5ml Eppendorf tube containing 300ml of the lysis buffer plus 10μg of proteinase K. After mixing thoroughly, the buffer was

then incubated for 60 minutes at 65°C and allowed to cool at room temperature for 30 minutes. The RNAase enzyme was added (1.5µl) and the lysate was incubated at 37°C for 30 minutes and was then placed in ice bath for 10 minutes before adding 100µl of the protein precipitation solution. Lysates were placed back into the ice bath for 5 minutes and then were vortexed for 20 seconds. After this, lysates were centrifuged at 13000 rpm for 10 minutes. The precipitated proteins were discharged and the DNA containing supernatant was transferred to a fresh 1.5ml Eppendorf tube containing 300µl of 100% isopropanol and 10µg of glycogen (DNA carrier). Samples were mixed gently and left at room temperature for 10 minutes. This was followed by 10-minute centrifugation at 13000 rpm. The precipitated DNA was left at the bottom of the tube and the supernatant was discharged. 300µl of 70% ethanol was added to the DNA and samples were centrifuged at 13000 rpm for 5 minutes. The supernatant was discharged and the precipitated DNA was dried at room temperature for 15 minutes. The DNA was re-suspended in 20µl of DNA hydration solution and was left overnight at room temperature. DNA was stored at -80°C for further use.

4.6.3. DNA extraction from paraffin-embedded tissue

DNA extraction from paraffin-embedded tissue was carried out using 10µm thick sections from the cerebellar cortex using a rotary microtome as described previously. Sections were mounted in glass slides and incubated overnight at 37°C.

Sections were de-waxed in two baths of xylene substitute of 5 minutes each and were re-hydrated as above. Once sections were re-hydrated, they were rinsed with TBS for 5 minutes. Sections were then scratched off the slide using a fresh scalpel

blade and tissue was collected in a clean Eppendorf. The DNA extraction was carried out as for buccal smears.

4.7 Polymerase Chain Reaction (PCR)

ApoE was genotyped using polymerase chain reaction as described by Hixon and Vernier (Hixon and Vernier 1990).

A single gene encodes ApoE. Three different alleles exist and this allelic variation gives rise to six different ApoE phenotypes. The *ε3* allele is the most frequent allele in most populations and it is regarded as the parent allele. The *ε2* and *ε4* alleles differ from *ε3* in a mutation each. These mutations allow the ApoE genotyping by PCR. A fragment of the exon 4 of the ApoE gene is amplified and the PCR product is cleaved by action of a restriction endonuclease such as Hha1, which recognizes the sequence guanidine-cytosine-guanidine-cytosine (GCGC) and cleaves the amplified DNA in all sites containing that sequence. In the 227 base pairs (bp) amplified fragment (Figure 4.1), the number of sequences in the amplified products varies for each allele. The size of cleaved products allows the genotyping. Two invariant small fragments of 16 bp and 18bp are common for all isoforms. The *ε3* allele has three other such sequences and cleaved products of 33bp, 48bp, and 91bp long are produced. The *ε2* allele is cleaved in four fragments one of 91bp long (as *ε3*), one of 81 bp long and the other two, which are common to all isoforms (invariant). The *ε4* allele has four variant fragments of 19bp, 34bp, 48bp and 72bp and the two invariant fragments (Figure 4.1).

The primers used in the present study were:

Sense: **5'-ACAGAATTCGCCCCGGCCTGGTA-3'**

Antisense: **5'-TCCAAGGAGCTGCAGGCGGCGC-3'**

Reagents:

Reagent	Final Concentration
Taq DNA polymerase (Life Technologies 18038-026) 5U/µl	1.25u
10X PCR buffer minus Mg (Life Technologies, Y02028) containing 200mM Tris-HCl, Ph 8.4 & 500 mM KCl	1X
Magnesium Chloride ($MgCl_2$) 50mM (Life Technologies Y 02016)	1mM
Dimethyl Sulfoxide (DMSO) (Sigma)	10%
Nucleotide mix aqueous solution (Amersham Pharmacia Biotech) containing 2mMATP, 2mMdCTP, 2mMdGTP , 2mMdTTP, Ph7.0	0.2mM each
Primer mix (Genosys)	0.5mM each
DNA	10%
Double distilled water (autoclaved) to 50µl	

Procedure

The PCR reaction was carried out in a total volume of 50µl. All the reagents at the final concentration were added to a 0.5ml Eppendorf tube and the remaining volume to 100µ; was obtained by adding 50µl of autoclaved mineral oil to prevent loss of samples due to evaporation.

Figure 4.1 Amplified PCR products and homozygous ApoE Genotypes

(a) Shows the amplified PCR product of 227bp.

(b) Different fragment sizes of ApoE homozygous cases after restriction enzyme digestion.

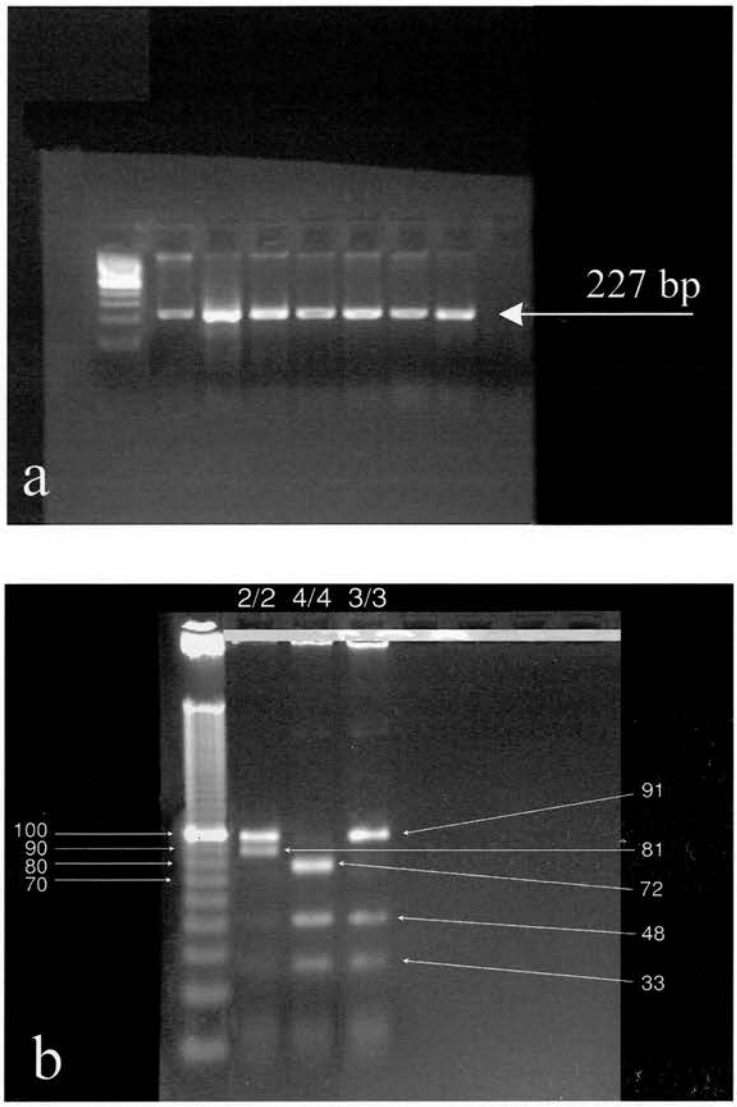

Figure 4.1

The PCR reaction was subjected to a thermal cycle of denaturation at 94°C for one minute and annealing of primers at 64°C for one minute and one final extension step at 72°C. The PCR product was amplified for 40 cycles.

The concentration of the reagents in the mix as well as the number of cycles for DNA amplification was optimized. The above-mentioned protocol consistently yielded better results for most of the cases. The PCR mix reaction was carried out using dedicated pipettes and aerosol resistant barrier tips in a dedicated laboratory.

4.8 Visualization of amplified PCR products

Visualization of the reaction by ethidium bromide using agarose gel electrophoresis was carried out prior to Hha1 digestion of the PCR product. Ethidium bromide exhibits fluorescence under ultraviolet light allowing the interpretation of the PCR results. The agarose gel was run for 30 minutes at 100v.

Reagents

10X TBE containing 108g of Tris base (AnalaR), 55g of boric acid (Molecular Biology Certified, Kodak) and 40ml of 0.5 M EDTA (Molecular Biology Certified Kodak). Volume adjusted to 1 liter with distilled water.

Agarose (Sigma). Final concentration 3%

Procedure

10ml of 10X TBE were diluted in 90ml of distilled water. 3g of Agarose were placed in a 500ml beaker, the 1X TBE solution was added and the mixture was stirred for two minutes. The mixture was subjected to microwave irradiation for one minute and 5µl of ethidium bromide was added. The gel was allowed to set at 4°C for one

hour prior to loading the PCR products. The gel was run for 30 minutes at 100v and the results were visualized under ultraviolet light.

4.9 Hha1 digestion

After the PCR reaction, 1μl (1unit) of Hha1 (Biolabs 20.000 u/ml) was added to the PCR reaction. The enzymatic digestion of the PCR products was obtained after overnight incubation at 37°C. The results were visualized by 4% metaphor agarose gel containing ethidium bromide. The metaphor-agarose gel is a high-resolution gel, which allows the identification of small DNA fragments.

Reagents

-10X TBE as above final concentration 1X

- Metaphor-agarose (FMC corporation) final concentration 4%

Ethidium bromide.

Procedure

To a 500ml beaker containing 100ml of 1X TBE, 4g of metaphor-agarose were added. The solution was left to soak for at least 15 minutes and was continuously stirred. Then the beaker was covered with cling film and the solution heated by microwave irradiation until it reached boiling. Occasional swirls were needed to ensure a thorough mix of the solution. Ethidium bromide (5μl) was added before the gel was allowed to set at 4°C for 1 hour. The gel was run for 2-3 hours and the results were analyzed under ultraviolet light.

4.10 Statistical Analysis

The statistical analysis was carried out using the commercial statistical program SPSS-10. Chi square method (X^2) was used for analysis of ApoE genotype and CD4/CD8+ T cell count frequencies as well as for the analysis of histological features and CD68 positivity between cases with and without ApoE 3\3 genotype. The significance level was set at $P<0.05$.

For the analysis of CD4/CD8 counts, the last recorded value (closest to the post-mortem examination) was used. Values were categorized by quartile. Each quartile had approximately the same number of cases. The statistical program used generated categories. Most of the histological features studied were recorded as binary variables (present or not present), except HIVE, which was recorded as an ordinal variable. Cognitive impairment (also an ordinal variable) was recorded as negative, mild, moderate and severe, while HIVE was recorded as negative, mild or florid (severe).

For comparisons concerning CD68 positivity, nine consecutive high power fields (20x) were measured for both white and gray matter of frontal, temporal hippocampus and thalamus sections. Fields were automatically selected by the image processing system using a microscope with automatized stage device. The number of microscopical fields measured assured avoidance of counting only microglial nodules in cases, in which that histological feature was present. The image processing system generated three sets of values namely percentage of positive pixels, total positive areas in pixels and total positive areas in microns. The average of the nine fields for each set of values was entered into the statistical program used

as independent variables. Analysis disclosed similar results for each set of values. Consequently, the total positive area in pixels was used for the analysis. After testing the results for normal distribution, a base 10 logarithmic transformation of the values was carried out in order to make values as close as possible to the normal distribution. Logarithm transformed data was used for analysis of variance (ANOVA). Differences between each pair of groups were disclosed using Tukey post-Hoc test. When comparing CD68 counts among different ApoE genotypes, cases of Group IV and V were analyzed together and ApoE was recoded as having or not having the 3\3 genotype. Values for total positive areas in pixels were categorized and the categories created were compared among the recoded ApoE genotypes and significant differences were obtained by X^2 tests with 2 degrees of freedom. Significance was set at $P<0.05$ level.

Chapter 5

RESULTS

5.1 Introduction

The aims of this study were to determine the influence of ApoE genotype and drug use in modifying HIV related disease progression. Different stages of the disease were studied due to the high rate of death among HIV positive asymptomatic individuals. The ApoE genotype of a cohort of HIV positive individuals (both drug users and non-drug users) compared to the ApoE genotype of HIV negative controls is first presented. The second part of the results section is concerned with the immune status of HIV positive cases in the last stages of HIV infection. This was assessed indirectly by comparing the last CD4 and CD8 counts. Comparisons between different risk groups and ApoE genotype both between and within risk groups were also carried out. The third part of the results describes some the neuropathological changes (which appeared to vary most between risk groups) and comparisons between these changes and ApoE genotype within and between groups. The final part of the results deals with comparisons of microglial counts in three different brain areas among different groups of cases, namely normal controls, HIV negative drug users, HIV positive drug users, and AIDS non-DU cases. Comparisons among ApoE genotype and microglial counts in the AIDS groups are also described.

5.2 ApoE Genotype Demographics of the Study Population

A total number of 312 cases divided into five groups were genotyped for ApoE. Table 5.2.1 shows the distribution of cases and mean age by group.

Table 5.2.1. Distribution of cases by group and mean age.

GROUP	NUMBER OF CASES	MEAN AGE
Normal controls (Group I)	64	39.3
HIV negative drug users (Group II)	82	34.1
HIV positive drug users, pre-symptomatic (Group III)	38	30.6
Drug users with AIDS (Group IV)	84	32.9
Non drug users with AIDS, MSM and blood transfusion, (Group V)	44	37.7
Scottish Population *	400	53

*(Cumming and Robertson, 1984). Key: MSM: Men who have sex with Men.

Group I included 64 normal controls. Group II comprised 82 HIV negative drug users. Group III had 38 HIV positive drug users in the pre-symptomatic stage. In group IV there were 84 HIV positive drug users with AIDS and group V included 44 cases of HIV positive non-drug users with AIDS. This last group consisted of 38 men who have sex with men (MSM) and 6 cases of transfusion associated HIV infection. Inclusion for AIDS groups was based either on CD4+ T cell counts less than 200 or

on the presence of AIDS defining illnesses. Published data for ApoE genotype in the normal Scottish population was used for comparisons. The latter was designated Group VI and included 400 cases.

The mean age for group VI was 53 years being older than the other groups (Table 5.2.1). There were no significant differences between the mean ages of the other five groups.

The HIV positive groups (Groups III to V) comprised 166 cases, of which 77% were male and 23% female. Gender was found significantly different between groups. This is explained mainly by the inclusion of MSM in group V.

5.3 ApoE Genotype

Groups III and IV had the same risk factor for contracting HIV and therefore they were analyzed together for the purpose of ApoE genotyping; this combined group is designated in further description as Group III/IV. The total number of HIV positive drug using cases was 122 with a mean age of 32.2 years.

Examples of the PCR results of ApoE genotype are displayed in Figure 5.1.

Table 5.3.1 shows the frequency of the six different ApoE genotypes by group, and table 5.3.2 shows the frequency of individual ApoE alleles among the groups studied. When comparisons of ApoE genotype between groups were subjected to statistical analysis, significant differences were found between group III/IV and group II ($X^2=5.619$, $p<0.018$), and group VI ($X^2=8.320$, $p<0.004$). This difference is explained mainly by an under-representation of ApoE $\varepsilon 3$ allele and an over-representation of ApoE $\varepsilon 2$ allele in group III/IV.

Figure 5.1 Example of PCR Results

Amplified PCR products of cases included in the present study are shown (a). Line 19 corresponds to the negative control.

Different ApoE genotypes of some of the studied cases are also shown (b). Line 11 corresponds to the negative control.

Group III/IV had a lower proportion of cases with the 3\3 ApoE genotype (45.9%) and an increased proportion of cases with the 3\4, 2\2 and 2\4 ApoE genotypes compared with any other group. The 3\2 ApoE genotype was found more frequently in group V but there were no cases with 2\2 or 2\4 genotype.

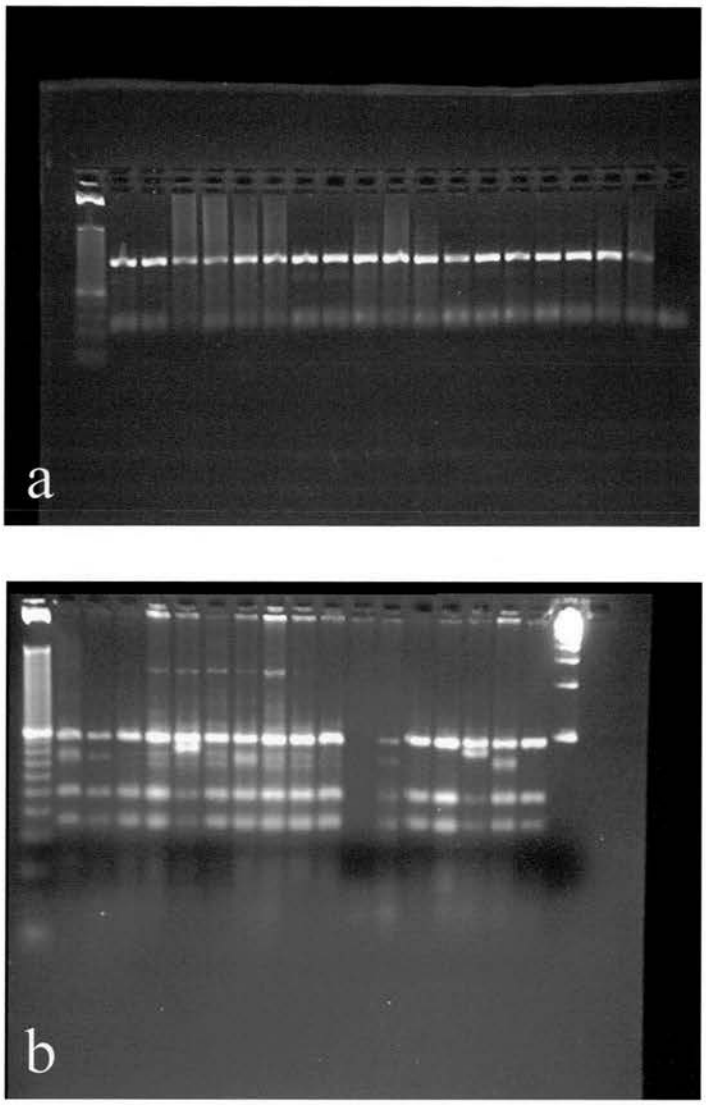

Figure 5.1

Table 5.3.1 Distribution of cases by ApoE genotype and group.

GROUP	APOE 3\3		APOE 3\4		APOE 3\2		APOE 2\2		APOE 4\4		APOE 2\4	
	N	%	n	%	n	%	N	%	n	%	n	%
Group I (normal controls) n=64	39	60.9	14	21.9	6	9.4	0	0	4	6.3	1	1.6
Group II (HIV negative DU) n=82	53	64.6	18	22.1	7	8.5	0	0	2	2.4	2	2.4
Group III (Pre-symptomatic HIV positive drug users) n=38	16	42.1	11	28.9	5	13.2	2	5.3	0	0	4	10.5
Group IV (Drug users with AIDS) n=84	40	47.6	24	28.6	12	14.3	3	3.6	2	2.4	3	3.6
Group III /IV (HIV positive DU) n=122	56	45.9	35	28.7	17	13.9	5	4.1	2	1.6	7	5.7
Group V (Non DU with AIDS) n=44	25	56.8	9	20.5	8	18.2	0	0	2	4.5	0	0
Scottish Population * n = 400	233	58.3	99	24.8	51	12.8	2	0.5	4	1	11	2.8

*(Cumming and Robertson, 1984). Key as in table 5.2.1.

Table 5.3.2 Frequency of ApoE alleles by group.

GROUP	APOE ε2	APOE ε3	APOE ε4
Group I n=64	5.4%	75.7%	18.9%
Group II n= 82	5.4%	79.8%	14.8%
Group III/IV n=122	14%	67.2%	18.8%
Group V n=44	9%	76.1%	14.9%
Group VI n=400	8.2%	77%	14.7%

Analysis of table 5.3.2 shows that the frequency for the ApoE *ε2* allele in group III/IV was 14% in contrast to group I and II with a frequency of 5.4% each for the same allele. This difference was significant (X^2= 5.756, $p<0.016$, X^2=7.017, $p<0.008$ respectively). The frequency of the same allele in group VI was 8.2% (X^2= 7.580, $p<0.006$), and in group V was 9%. Comparisons between group III/IV and group V also showed differences in ApoE distribution but this did not reach significance almost certainly due to the sample size of Group V.

The frequency for the ApoE *ε3* allele for group III/IV was 67.2% and for group II was 79.8% and was 77% for group VI. These differences were significant (X^2= 7.392, $p<0.007$ and X^2=9.695, $p<0.002$ respectively).

No significant differences were found in comparisons of frequencies for ApoE *ε3* alleles between group III/IV, and groups I and V, nor in any of the comparisons for ApoE *ε4* allele.

All comparisons between group VI and groups I, II, and V were not significant.

Comparisons between all HIV positive groups taken together (Groups III/IV, and V) with HIV negative groups (Groups I, II and VI), showed significant differences for the ApoE *ε2* allele (X^2=9.162, $p<0.002$), for the ApoE *ε3* allele (X^2=8.218, $p< 0.004$) and for the six ApoE genotypes (X^2=7.382, $p<0.007$) frequencies. The over-

representation of ApoE *ε2* allele and the under-representation of ApoE *ε3* allele were the main differences found, but this is almost certainly because of the relative high frequency of ApoE *ε2* in the drug using group (Group III/IV).

In summary, differences in ApoE genotype were found between group III/IV and all other groups. There was an under-representation of ApoE *ε3* and an over-representation of ApoE *ε2* in the HIV positive drug users, which was still evident when the other HIV positive group (Group V) was included and all HIV positive cases were analyzed together. The inclusion of Group V did not cause the significant findings obtained for Group III/IV to disappear.

5.4 Comparisons of CD4 and CD8 T cell counts and ApoE genotype in AIDS cases

Groups IV and V included all AIDS cases studied. Only cases with CD4+ T cell counts less than 200/cells/μl of blood were used for comparisons. Eight cases with AIDS defining illnesses (6 in Group IV and 2 in Group V) which either had CD4 counts above 200 or which had no record for CD4 counts were excluded from the analysis. Seventy-eight and 42 cases in group IV and V respectively had the last CD4+ T cell count lower than 200 cells, yielding a total number of 120 cases in these two groups. Four cases of Group V had no CD8+ T cell counts. The mean age was 33 years for group IV cases and 38 years for cases for group V.

For the group as a whole (drug users and non-drug users with AIDS) mean and median values for CD4 and CD8 counts by ApoE genotypes are displayed in table 5.4.1.

Table 5.4.1 Mean and median values for CD4 and CD8 counts by ApoE genotype

ApoE Genotype		CD4	CD8
	Mean	34	358
3\3	Median	10.5	192
n=58	Missing	0	2
	Mean	54.4	359
3\4	Median	18	230
n=32	Missing	0	1
	Mean	54.3	480
3\2	Median	30	400
n=20	Missing	0	0
	Mean	58	174
2\2	Median	3	170
n=3	Missing	0	0
	Mean	20.2	488
4/4	Median	17.5	450
n=4	Missing	0	1
	Mean	72.6	1084
2\4	Median	87	1034
n=3	Missing	0	0

Cases homozygous for ApoE *ε3* allele had lower mean and median values for CD4 counts than the cases heterozygous for the same allele. CD4 mean values for 3\4 and 3\2 genotypes were similar, but median values were higher for 3\2 than for 3\4 and

these in turn were higher than for 3\3. Cases with ApoE 3\2 genotype had a three-fold higher median value for CD4 cell counts than 3\3 cases.

CD8 counts were not available in 4 cases.

Again for CD8 counts, 3\2 cases had higher mean and median values than 3\4 and 3\3. There was not a large difference in mean and median values for CD8 counts between 3\4 and 3\3 cases. Cases with ApoE 3\2 genotype had two-fold increase in median values for CD8 cell counts when compared with 3\3 cases.

The ApoE genotype for each group of AIDS cases with known CD4 cell counts of less than 200/μl blood is displayed in table 5.4.2.

Thirty-four of the cases of Group IV accounting for 43.5% of all cases in that group and 24 (57.1%) of the cases of Group V were 3\3 ApoE genotype. 24 cases (30.7%) of Group IV and 8 cases (19%) of Group V were 3\4 ApoE genotype. 12 (15.3%) of the cases of Group IV and 8 (19%) of the cases of Group V had a 3\2 ApoE genotype. There were 3 cases with 2\2 and 3 cases with 2\4 ApoE genotypes in Group IV and neither of these genotypes were found in Group V. Two cases in each group, had 4\4 ApoE genotype.

Table 5.4.3 shows the minimum, maximum, mean and median values for CD4, CD8 and CD4/CD8 ratio by group. The median value divides the results in half, that is half of the values are below and half are above the median value.

Table 5.4.2 Distribution of ApoE genotype by group in AIDS patients with known CD4/CD8 counts

GROUP	3\3		3\4		3\2		2\2		4\4		2\4		TOTAL	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
Group IV n=78	34	**43.5**	24	**30.7**	12	**15.4**	3	**3.9**	2	**2.5**	3	**3.9**	78	**99.9**
Group V n=42	24	**57.1**	8	**19**	8	**19**	0	**0**	2	**4.8**	0	**0**	42	**99.9**
Total	58		32		20		3		4		3		120	

Table 5.4.3. Minimum, maximum, mean and median values for CD4, CD8, CD4/CD8 ratio by group.

GROUP		CD4	CD8	CD4/CD8 RATIO
	Number	78	78	78
IV	Missing	0	0	0
	Minimum	1	27	0.00
	Maximum	199	2611	0.1
	Mean	**57.85**	**468.01**	**0.14**
	Median	**23**	**273**	**0.092**
	Number	42	38	38
V	Missing	0	4	4
	Minimum	1	15	0.00
	Maximum	160	769	0.37
	Mean	**17.95**	**251.26**	**0.069**
	Median	**6.5**	**182.5**	**0.038**

Analysis of table 5.4.3 showed that all values were higher for group IV.

The mean value for CD4 counts for group IV cases was 57.85cells/μl and for group V cases was 17.95cells/μl. Median value for CD4 counts in the 42 cases of group V was 6.5 cells/μl, while for the 78 cases of Group IV it was 23 cells /μl. The median values for CD8 and CD4/CD8 ratio for group V were 182.5 and 0.038 respectively, and for group IV cases were 273 and 0.092 respectively.

CD4, CD8 and CD4/CD8 ratio mean, median, minimum and maximum values by group and ApoE genotype are displayed in table 5.4.4.

Table 5.4.4. Descriptive statistics by Group and ApoE genotype

APOE GENOTYPE		CD4		CD8		CD4/CD8	
		Group IV	Group V	Group IV	Group V	Group IV	Group V
3\3 n=58	Number	34	24	34	22	34	22
	Missing	0	0	0	2	0	2
	Min	1	1	27	15	0.01	0.00
	Max	160	160	2530	769	0.43	0.37
	Mean	**43.97**	**19.7**	**425**	**256.2**	**0.12**	**0.075**
	Median	**19**	**4.5**	**192**	**196**	**0.09**	**0.036**
3\4 n=32	Number	24	8	24	7	24	7
	Missing	0	0	0	1	0	1
	Min	1	2	52	40	0.00	0.02
	Max	199	50	2611	455	0.57	0.14
	Mean	**66.95**	**16.62**	**420.4**	**149.4**	**0.15**	**0.078**
	Median	**34**	**7**	**273**	**119**	**0.10**	**0.075**
3\2 n=20	Number	12	8	12	8	12	8
	Missing	0	0	0	0	0	0
	Min	2	1	85	141	0.02	0.01
	Max	156	64	1687	725	0.35	0.22
	Mean	**79.75**	**16.12**	**577**	**333.3**	**0.16**	**0.049**
	Median	**85**	**7.5**	**480**	**296**	**0.15**	**0.028**
2\2 n=3	Number	3	0	3	0	3	0
	Missing	0	0	0	0	0	0
	Min	1	0	143	0	0.01	0
	Max	170	0	210	0	1	0
	Mean	**58**	**0**	**174**	**0**	**0.34**	**0**
	Median	**3**	**0**	**170**	**0**	**0.014**	**0**
4\4 n=4	Number	2	2	2	1	2	1
	Missing	0	0	0	1	0	1
	Min	22	6	450	198	0.03	0.03
	Max	40	13	315	198	0.09	0.03
	Mean	**31**	**9.5**	**632**	**198**	**0.05**	**0.03**
	Median	**31**	**9.5**	**632**	**198**	**0.05**	**0.03**
2\4 n=3	Number	3	0	3	0	3	0
	Missing	0	0	0	0	0	0
	Min	23	0	440	0	0.05	0
	Max	108	0	1779	0	0.1	0
	Mean	**72.6**	**0**	**1080**	**0**	**0.068**	**0**
	Median	**87**	**0**	**1034**	**0**	**0.50**	**0**

Table 5.4.4 shows that again, nearly all values for group IV cases were higher than for group V cases with the same ApoE genotype.

For group IV, 34 cases with the ApoE 3\3 genotype had a mean CD4 count of 43.97 and a median of 19 cells. Increasingly higher values were observed for other ApoE *ε3* heterozygous genotypes such as 3\4 and 3\2. Median values were also higher. In 14 cases with ApoE genotype 3\4 the median was 34 and in 12 cases with ApoE genotype 3\2 the median was 85 cells. Notably, lower mean values for CD4 counts were observed for cases with ApoE 4\4, when compared with any other cases of the same group with different ApoE genotype, although the median for 4\4 genotype (31) was higher than that for 3\3 drug users (19). However, the number of cases that have no *ε3* allele is small.

Group V cases with ApoE 3\3 genotype (homozygous for *ε3*), had higher mean values for CD4 but the median value was the lowest when compared with the other ApoE genotypes in that group. It should be noted that no 2\2 and 2\4 cases were present in Group V, indicating a greater variation in CD4 counts in non-drug using cases homozygous for ApoE *ε3* allele.

Interestingly, the five other homozygous individuals for ApoE genotype in Group IV, namely 2\2 and 4\4, had slightly lower values for mean and median than all heterozygous cases (3\4, 3\2 and 2\4). For cases with ApoE 4\4 genotype, slightly lower mean and median values were observed when compared with other cases in this group with different ApoE genotype.

Similar results were obtained for CD8 counts and CD4/CD8 ratio (table 5.4.4). All mean values for CD8 counts were higher for Group IV cases than for Group V cases

of the same ApoE genotype. The median values for cases with 3\3 ApoE genotype of both groups were almost the same. For Group IV, cases with 3\4 and 2\2 ApoE genotype had lower mean values for CD8 counts than cases with 3\3 ApoE genotype. The median value, however, of cases with 3\4 ApoE genotype was higher that the median value of cases with 3\3 ApoE genotype. The same was not observed in the three cases of group IV with 2\2 ApoE genotype.

The CD4/CD8 ratio was higher in Group IV than in Group V for all ApoE genotypes.

In summary, cases of Group V had lower numbers of CD4, CD8 cells and CD4/CD8 ratio when compared to cases of Group IV. Cases with 3\3 ApoE genotype had lower values for CD4 and CD8 cells than cases of the same risk group with 3\4, and 3\2 ApoE genotypes. There were fewer homozygous cases apart from 3\3 cases (2\2 and 4\4) and they had slightly lower CD4 and CD8 counts than 3\3 cases.

In order to assess the relationship between CD4, CD8 counts and CD4/CD8 ratio values, with ApoE genotype for the whole group of AIDS cases, and to assess differences between groups IV and V, CD4, CD8 and CD4/CD8 ratio values were divided in quartiles. Analysis was first undertaken based on ApoE genotype of all AIDS cases (drug users and non-drug users). Significant associations between ApoE genotype or ApoE alleles and CD4 and CD8 counts in each group are also mentioned. The final part of this section refers to findings regarding CD4 and CD8 counts and CD4/CD8 ratio between risk groups independently from ApoE genotype.

The first quartile included cases with values below the 25^{th} percentile, the second quartile, cases below the 50^{th} percentile and the 3^{rd} and 4^{th} quartiles include cases

with values below and above the 75th percentile respectively. Table 5.4.5 shows values for each quartile. 28 cases were included in the first quartile and all had CD4 values below 4 cells/μl. The second quartile had 32 cases with CD4 values between 4-17 cells/μl. There were 31 cases in the third quartile with CD4 values ranging between 18-80 cells/μl. The fourth quartile included 29 cases with CD4 values ranging from 81-199 cells/μl.

Table 5.4.5 Distribution of quartile values for CD4, CD8 and CD4/CD8 ratios for all AIDS cases

QUARTILES	CD4		CD8		CD4/CD8 RATIO	
	Value	**Mean**	Value	**Mean**	Value	**Mean**
1 n=28	1-3	**1.5**	15-103	**63.4**	0-0.03	**0.01**
2 n=32	4-17	**8.8**	109-246	**160.0**	0.03-0.08	**0.04**
3 n=31	18-80	**38.8**	260-455	**353.3**	0.08-0.16	**0.11**
4 n=29	81-199	**129.5**	520-2611	**1001.7**	0.16-1	**0.3**

The distribution of cases with CD8 counts by quartile was similar to that for CD4 but there were 4 cases with missing values. Cases with CD8 counts lower than 103 cells/μl were included in the first quartile (28 cases). 29 cases with CD8 counts between 109-246 cells/μl and 30 cases with counts ranging between 260-455 cells/μl were included in the second and third quartiles respectively and the fourth quartile included 29 cases with more than 455 CD8 cells/μl.

Table 5.4.6 shows the distribution of CD4, CD8 and CD4/CD8 ratio by quartile and ApoE genotype in all AIDS cases.

Table 5.4.6. Distribution of CD4, CD8 and CD4/CD8 ratio values by quartiles and ApoE genotype for all AIDS cases.

QUARTIL.	3\3			3\4			3\2			2\2			4\4			2\4		
	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8
1 n=28	15	18	14	9	8	8	2	2	5	2	0	2	0	0	0	0	0	0
2 n=32	17	13	13	7	9	6	6	3	6	0	3	0	2	1	2	0	0	0
3 n=31	16	12	16	6	8	9	6	8	8	0	0	0	2	1	1	1	1	2
4 n=29	10	13	13	10	6	8	6	7	7	1	0	1	0	1	0	2	2	1
Total n=120	**58**	**56**	**56**	**32**	**31**	**31**	**20**	**20**	**20**	**3**	**3**	**3**	**4**	**3**	**3**	**3**	**3**	**3**

Analysis of table 5.4.6 showed that 44.8% (26 cases out of 58) of the cases with ApoE 3\3 genotype had CD4 counts above the 50th percentile (third and fourth quartiles). 50% (16 cases) and 60% (12 cases) of the cases with ApoE 3\4 and 3\2 genotypes, also had CD4 counts above the 50th percentile. For the fourth quartile however, 17.2% of the cases, 10 out of 58 with 3\3 ApoE genotype had CD4 counts between 80 and 199 in contrast to 31% and 30% (10 and 6 cases respectively) with 3\4 and 3\2 ApoE genotypes.

The association between CD4 counts and ApoE genotype did not reach significance, neither analyzing the data for both groups individually nor both groups taken together. Drug user cases with ApoE genotype 3/3 had a tendency towards lower CD4 counts but this was not statistically significant. With the exception of two cases with ApoE genotype 4\4, all cases with ApoE genotypes different from 3\3 had higher CD4 values. For non-drug users (Group V) the opposite effect is apparent.

Regarding CD8 counts, 44.7%, (25 cases) 45.2%, (13 cases) and 75% (15 cases) of the cases with ApoE genotypes 3\3, 3\4 and 3\2 respectively had CD8 counts above the 50th percentile (third and fourth quartiles). For other genotypes, 2 out of 3 cases and all three cases with 4\4 and 2\4 ApoE genotypes respectively had CD8 counts above the 50th percentile. A significant association between ApoE genotype (all six possible combinations of alleles) and CD8 counts was found (X^2=12.236, $p<0.03$). When analyzing data for both groups individually, the association was only significant in Group IV (X^2=12.052, $p<0.03$).

Of all AIDS cases with one or two ApoE $\varepsilon 2$ alleles (n=26), 18 (69.2%) had CD8 counts above the 50th percentile (9 cases each in the third and fourth quartiles) and

only 2 cases (7.6%) were in the first quartile. A significant association was found between ApoE *ε2* allele and CD8 counts (X^2=4.524, p<0.03) even after adjusting for group and age. Incidentally, for group V, almost all cases with at least one ApoE *ε4* allele had CD8 counts below the 50th percentile (7 cases out of 8). Thus a negative association between CD8 counts and ApoE *ε4* allele was found for cases in Group V (X^2=4.812, p<0.02).

In summary, these findings showed that AIDS cases with ApoE genotype 3\3 tend to have lower CD4 and CD8 counts, but the association was significant only for CD8 counts. An opposite effect of ApoE *ε2* and *ε4* alleles for CD8 counts was observed, with ApoE *ε2* being associated with higher CD8 counts and ApoE *ε4* with lower CD8 counts, as for ApoE *ε3*. This is borne out by inspection of table 5.4.4

Table 5.4.7 shows the distribution of ApoE genotyped cases for each quartile by groups. This table shows that for Group IV, 14 cases out of 78 had CD4 values within the first quartile, 16 in the second, 20 in the third and 28 in the fourth. For Group V, 14 cases out of 42 were in the first quartile and 16, 11 and 1 cases in the second, third and fourth quartiles respectively. Regarding CD8, for Group IV, 18 cases were in the first quartile, 19 in the second quartile, 16 in the third and 25 in the fourth quartile and for Group V 10, 10, 14 and 4 cases were in the first, second, third and forth quartiles respectively.

Regarding CD4/CD8 ratios, for Group IV, 15 cases were in the first quartile and 13 in the second quartile, 24 in the third quartile and 26 in the fourth quartile. For Group V, there were 14 cases in the first and second quartiles each and 5 cases in the third and fourth quartiles each. Thus, 61.5% (48 cases) of the cases in group IV had CD4

counts above the 50th percentile (third and fourth quartiles), in contrast to only 28.5% of the cases in group V. This was significant (X^2=11.769, p<0.001). Similarly, 64.1% of the cases of group IV (50 cases) had CD4/CD8 ratio values above the 50th percentile in contrast to 26.3% (10 cases) of group V cases. (X^2=8.622, p<0.002).

For CD8 counts 52.5% and 47.3% of the cases of groups IV and V respectively had counts above the 50th percentile, but this did not reach significance.

In summary, Group IV had higher values for CD4 and CD8 counts than Group V. Significant associations between risk group (drug use and non-drug use) and CD4 and CD4/CD8 ratios were obtained even after adjusting for age. Higher CD8 counts were associated with ApoE *ε2* after adjusting for group and age. ApoE genotype *ε2* and *ε4* appear to have opposite effect on CD8 counts.

5.5 ApoE and Neuropathological findings

5.5.1 Study Population Demographics.

Detailed histological examination of the brain was carried out in 78 AIDS cases. Of those, 49 were in Group IV and 29 cases were in Group V. The mean age for group IV cases was 32.16 years and for group V was 37.6 years. In Group IV, 15 cases were female and 34 cases were male. For group V there was only one female case. All of these cases had CD4 counts below 200 cells/μl.

Detailed histological examination was also carried out in 19 pre-symptomatic HIV positive cases. The mean age for this group of cases (Group III) was 32 years. 13 cases were males and 6 were females.

5.5.2 Histological Parameters Investigated

The histological parameters were scored as present or absent and if present were graded according to degree of severity. The features were graded as absent (-), or mild (+) if they were rarely present and/or were present in only one section of the brain, or moderate/severe (++) if they were present frequently and/or in two or more sections of the brain. Histological parameters assessed, included:

A) HIV encephalitis (HIVE)

Although many of the histological findings listed below are associated with HIVE, the presence of multinucleated giant cells (MNGC) or the immunocytochemical identification of HIV antigens in the brain tissue was required for its diagnosis (Budka et al, 1991). Multinucleated giant cells were seen around blood vessels in white matter, cortical grey matter and subcortical grey matter and occasionally, they were found in both (grey and white matter). Usually other inflammatory cells including microglia or macrophages were seen accompanying MNGC. The number of these cells varied from case to case. In some cases numerous MNGC were seen in the same section or they were seen less frequently in several histological sections of the same case. Those cases were regarded as having florid HIVE. In other cases however, MNGC were sparse and/or only seen in one histological section. Those cases were regarded as having mild

HIVE. In most HIVE cases other histological features such as microglial nodules or white matter damage were also present (See Fig. 5.2).

Table 5.4.7. Distribution of cases in Group IV and V by quartile and ApoE genotype

Quartile	ApoE	CD4				CD8				CD4/CD8 Ratio			
		Group IV		Group V		Group IV		Group V		Group IV		Group V	
		#	%	#	%	#	%	#	%	#	%	#	%
1	3\3	5	**6.4**	10	**23.8**	11	**14.1**	7	**18.4**	5	**6.4**	9	**23.6**
	3\4	6	**7.6**	3	**7.1**	5	**6.4**	3	**7.8**	7	**8.9**	1	**2.6**
	3\2	1	**1.3**	1	**2.3**	2	**2.6**	0	**0**	1	**1.3**	4	**10.5**
	2\2	2	**2.6**	0	**0**	0	**0**	0	**0**	2	**2.6**	0	**0**
	4\4	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	2\4	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	Total	14	**18**	14	**33.3**	18	**23**	10	**26.3**	15	**19.2**	14	**36.8**
2	3\3	11	**14.1**	6	**14.2**	9	**11.5**	4	**10.5**	6	**7.6**	7	**18.4**
	3\4	4	**5.1**	3	**7.1**	6	**7.6**	3	**7.8**	3	**3.8**	3	**7.9**
	3\2	1	**1.3**	5	**11.9**	1	**1.3**	2	**5.2**	3	**3.8**	3	**7.9**
	2\2	0	**0**	0	**0**	3	**3.8**	0	**0**	0	**0**	0	**0**
	4\4	0	**0**	2	**4.7**	0	**0**	1	**2.6**	1	**1.3**	1	**2.6**
	2\4	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	Total	16	**20.5**	16	**38**	19	**24.3**	10	**26.3**	13	**16.6**	14	**36.8**
3	3\3	9	**11.5**	7	**16.5**	4	**5.1**	8	**21**	14	**18**	2	**5.2**
	3\4	4	**5.1**	2	**4.9**	7	**8.9**	1	**2.6**	6	**7.6**	3	**7.9**
	3\2	4	**5.1**	2	**4.9**	3	**3.8**	5	**13.1**	2	**2.6**	0	**0**
	2\2	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	4\4	2	**2.6**	0	**0**	1	**1.3**	0	**0**	1	**1.3**	0	**0**
	2\4	1	**1.3**	0	**0**	1	**1.3**	0	**0**	1	**1.3**	0	**0**
	Total	20	**25.6**	11	**26.1**	16	**20.5**	14	**37**	24	**30.7**	5	**13.1**
4	3\3	9	**11.5**	1	**2.3**	10	**13**	3	**7.8**	9	**11.5**	4	**10.5**
	3\4	10	**13**	0	**0**	6	**7.6**	0	**0**	8	**10.2**	0	**0**
	3\2	6	**7.8**	0	**0**	6	**7.6**	1	**2.6**	6	**7.6**	1	**2.6**
	2\2	1	**1.3**	0	**0**	0	**0**	0	**0**	1	**1.3**	0	**0**
	4\4	0	**0**	0	**0**	1	**1.3**	0	**0**	0	**0**	0	**0**
	2\4	2	**2.3**	0	**0**	2	**2.6**	0	**0**	2	**2.6**	0	**0**
	Total	28	**35.8**	1	**2.3**	25	**32**	4	**10.5**	26	**33.3**	5	**13.1**
Total		78	**99.8**	42	**99.7**	78	**99.8**	38	**99.9**	78	**99.9**	38	**99.8**

B) Perivascular mononuclear infiltration

This feature was defined as the presence of mononuclear cells (lymphocytes) around blood vessels (within the Virchow-Robin space). Variation in the number of these inflammatory cells was observed according to different blood vessel sizes in each case. This feature was present in almost all pre-symptomatic HIV cases (Group III) and showed variation among AIDS cases (Groups IV and V). Most of the inflammatory cells had the morphology of lymphocytes but macrophages were also found around blood vessels (See Fig. 5.3).

C) Microglial Nodules.

Microglial nodules consisted of aggregates of hypertrophied microglial cells in white matter or amongst neurons in gray matter. The number and size of the cells forming the microglial nodules varied. This change was observed mainly in association with productive HIV infection of the CNS (multinucleated giant cells or HIV p24 immunoreactivity) and/or with cytomegalovirus infection, but in some cases no obvious association with either of these infections was observed (see Fig. 5.2).

D) Neuronophagia

Neuronophagia was considered present when hypertrophied microglial cells were observed closely applied to (around) abnormal neurons of cortical and/or subcortical grey matter. The extent of this feature also varied from case to case. In some cases it was observed as clusters of affected neurons within a particular area. Those cases were graded as having severe (++) neuronophagia. The areas most affected were the temporal hippocampus and basal ganglia, substantia nigra and, the inferior olivary

nuclei. In other cases neuronophagia was seen occasionally in one or several sections of these same brain areas. The latter cases were graded as mild/moderate (+) (see Fig. 5.3).

E) Axonal balloons

Axonal balloons were seen in routinely stained sections, as well delineated eosinophilic, round structures of various sizes in the white matter in all areas of the CNS, including the spinal cord. In histological preparations immunohistochemically stained for β amyloid precursor protein (APP), they are easily seen and were usually surrounded by other abnormal and immunopositive axons.

F) Vascular damage

This was considered to be present when infarcts (usually small) were seen. The size and distribution varied from case to case. Occasionally inflammatory cells in blood vessel walls were also seen.

G) Mineralization

This feature in the CNS took the form of small areas of mineralization present in blood vessel walls or in the neuropil without any associated structure. They were seen most frequently in basal ganglia sections. The extent of this change varied between cases.

H) Cytomegalovirus infection (CMV)

Cytomegalovirus infection was considered to be present when basophilic intranuclear and intracytoplamatic inclusion bodies surrounded by a clear halo were seen, mostly

in otherwise enlarged macrophages or endothelial cells. CMV can infect any cell type in the CNS. Other histological changes seen in association with these infected cells included gliosis, microglial nodules and perivascular cuffs of lymphocytes. Frequently, inclusion-bearing cells were seen in necrotic areas (the most severe form), usually around the ventricles, but they were also seen in subcortical grey matter, cerebellar cortex, spinal cord and in dorsal root ganglia. The extent of accompanying inflammatory infiltrate varied widely between different CNS areas of the same case and between cases. Immunohistochemical confirmation of CMV was available in some cases.

Although other opportunistic infections were seen occasionally, their frequency did not merit a separate analysis. Consequently, they were not used for any comparison.

I) CNS lymphoma

Lymphoma was considered present when extensive, ill-defined infiltration of blood vessel walls by pleomorphic, abnormally large neoplastic lymphocytes were seen. Reticulin stains disclosed the distinctive pattern of blood vessel wall invasion. Mitotic figures were frequently seen. In one case of metastatic lymphoma (in Group IV) neoplastic cells were seen in the meninges. In the other seven cases (2 in Group IV and 5 in Group V) the neoplastic infiltration was restricted to the brain tissue. In all cases the neoplastic cells were large, pleomorphic with an evident nucleoli (Fig. 5.4). These lymphomas were classified as diffuse large B cell lymphomas according to the European-American lymphoma (REAL) classification (Mason and Harris 1999).

J) White matter damage

A degree of white matter damage was almost invariably present in all cases. This ranged from focal vacuolization associated with mononuclear inflammatory cells (including macrophages) and/or myelin pallor in myelin stained sections, to more severe diffuse involvement of deep white matter characterized by reactive astrocytes, with some degree of oedema and inflammatory infiltrate. Severe forms were frequently seen in cases with microglial nodules and multinucleated giant cells. Because this feature was present almost universally it was not used for comparisons.

K) Peripheral nerve inflammation

This was considered present when lymphoid cells were seen around endoneural blood vessels (Fig. 5.4).

5.5.3 Neuropathological changes and ApoE status in pre-symptomatic HIV positive drug users (Group III).

The defining criteria for initial inclusion of cases in group III were the presence of more than 200 CD4 +T cells/μl of blood and in cases without CD4 counts, the apparent lack of AIDS defining illnesses at full postmortem examination. Detailed histological examination was undertaken in 19 cases in this group.

In 12 cases, CD4 counts were available. The mean value for CD4 counts was 339.75 with a median of 253.5. 11 cases also had CD8 counts and the mean and median values were 784.9 and 510 respectively.

Figure 5.2 Multinucleated Giant cells and Microglial Nodules

Multinucleated giant cells (the hallmark of HIV encephalitis) are shown.

Multiple giant cells are seen together with macrophages and reactive astrocytosis (a).

A single multinuclated giant cell in the white matter (b).

A microglial nodule in the white matter together with some multinucleated giant cells (c). These two histological features were seen frequently associated.

(H and E, magnification (a) X200; (b) X400; (c) X100).

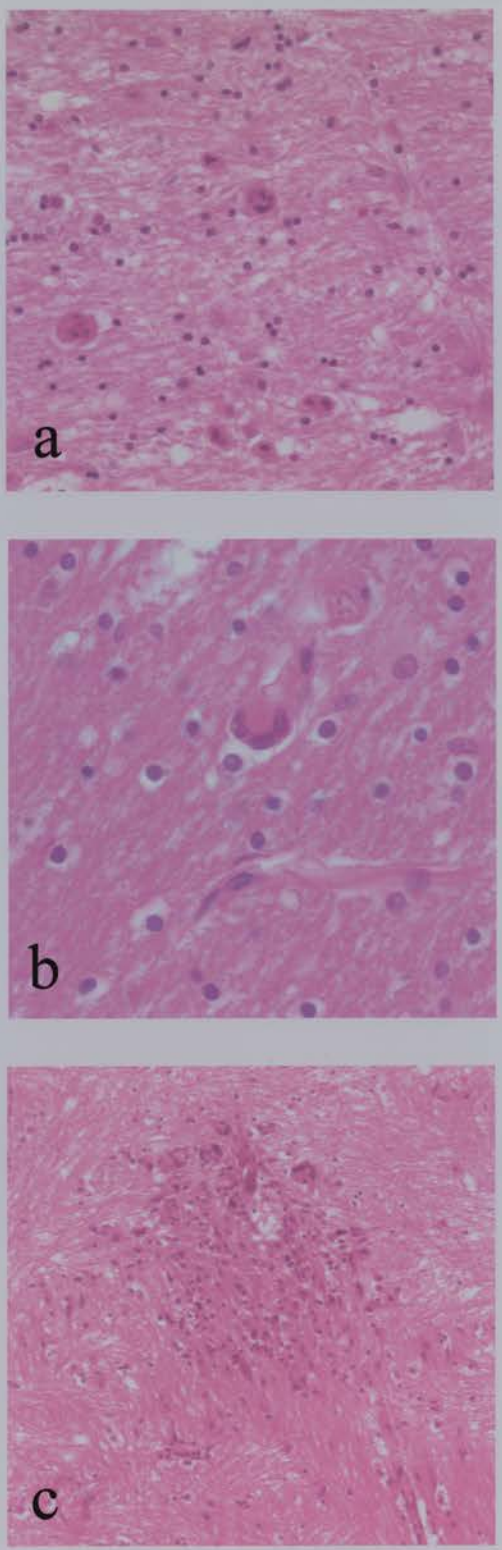

Figure 5.2

Figure 5.3 Perivascular cuffing of lymphocytes and Neuronophagia

Perivascular cuffing of lymphocytes (a). Perivascular lymphocytic infiltrate was the most frequent histological feature of HIV positive pre-symptomatic cases. This feature was also observed in AIDS cases.

Neuronophagia in basal ganglia (b).

Neuronophagia of pigmented neurons in the substantia nigra (c).

This histological feature was frequently seen in cases with HIVE.

(H and E, magnification (a) X200; (b) X400; (c) X400).

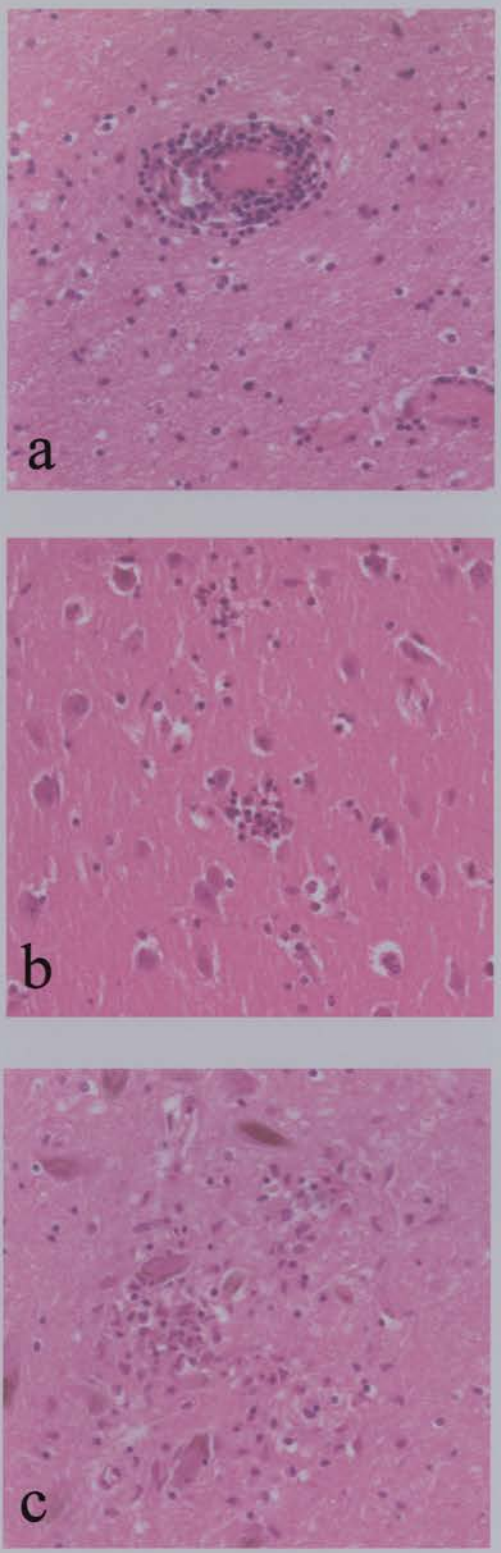

Figure 5.3

Figure 5.4 Peripheral nerve inflammation and Primary CNS lymphoma

Peripheral nerve inflammation (a). Lymphocytes and macrophages surrounding endoneural blood vessels (H and E X200).

Primary central nervous system lymphoma (b).

The perivascular spread of highly anaplastic tumour cells.

All lymphoma cases histologically studied showed similar tumour cell morphology and pattern of spread, (H and E X200).

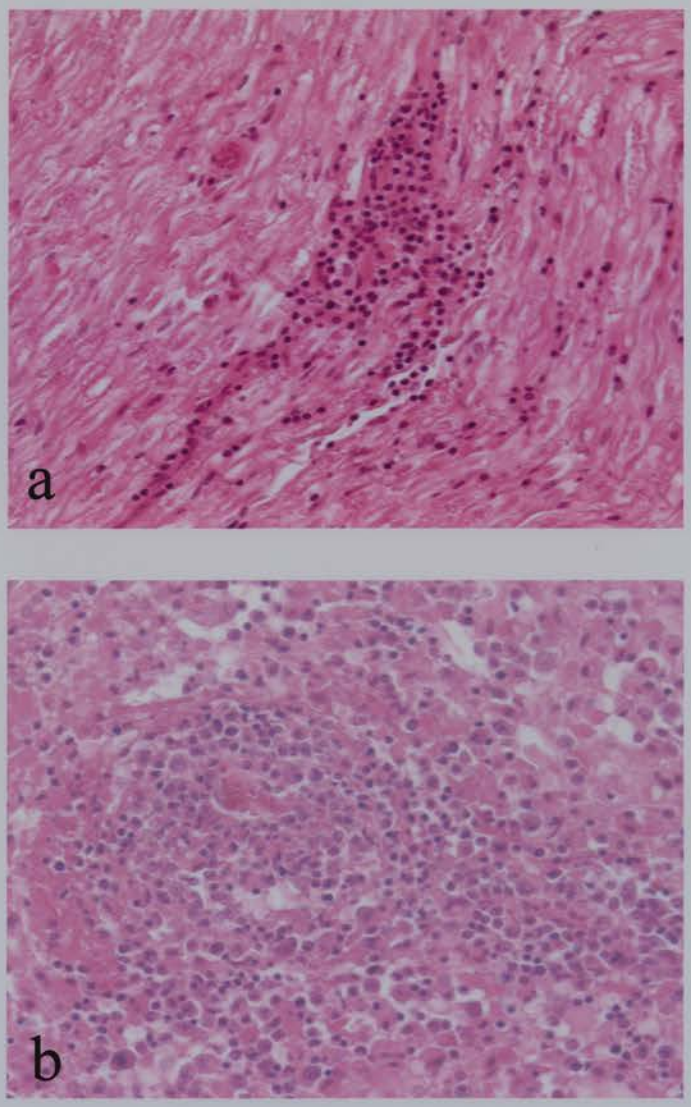

Figure 5.4

The distribution of ApoE genotype in these cases is shown in table 5.5.3.1

Numbers are very small, but data is shown for completeness.

Table 5.5.3.1 Distribution of cases in Group III by ApoE genotype

3\3		4\4		3\2		2\2		4\4		2\4		TOTAL
N	%	N	%	N	%	N	%	N	%	N	%	
7	36.8	4	21.1	3	15.8	2	10.5	0	0	3	15.8	19/100%

The most prominent histological change in this group was the presence of a lymphocytic inflammatory infiltrate around blood vessels. This was seen in 18 cases (94.7%). A surprising finding was that 4 cases (21%) had occasional MNGC, but these cases had CD4 counts slightly higher than 200cells/μl (mean value 234.75). On the basis of CD4 counts these cases would be classed as pre-AIDS but the presence of HIVE is a defining criterion for AIDS. The ApoE genotype of these cases was 3\3 in three cases and 3\4 in one. These four cases should be reclassified as AIDS cases and were not included in further analysis.

Microglial nodules were seen in the grey and white matter of the spinal cord only in two cases. They were also seen in the basal ganglia in one case, in the thalamus in one case, in the white matter of the medulla in one case, in the central white matter, white matter of the temporal hippocampus and in the basal ganglia in another case and in the pons, medulla and basal ganglia in one case. This accounted for a total of 7 cases (46.7%) with this finding. The mean value for CD4 T cell counts for these cases was 270 cells/μl.

Neuronophagia was seen in the mamillary bodies and basal ganglia in one case.

Peripheral nerve samples were available for two cases, and lymphocytes around endoneural blood vessels were seen in both of them.

Perivascular lymphocytes were also seen around blood vessels of the cauda equina in 7 cases, in the dorsal root ganglia in 5 cases and in the trigeminal nerve ganglion in one case.

Axonal balloons were seen in 4 cases. Affected anatomical areas included the white matter of the vermis in one case, the white matter of medulla in one case, the basal ganglia in one case and in the white and grey matter of the spinal cord in one case. The mean value for CD4 T cell counts of these cases was 371 cells/μl blood.

5.5.4 Neuropathological findings in AIDS cases

Histological examination of brain was carried out in 78 cases, 49 of them were in Group IV (drug users) and 29 in Group V. Information on cognitive impairment was also included for each case.

The pathological changes for each case are displayed in appendix III. The main differences between the groups included the presence and degree of cognitive impairment, HIVE and CMV infection. A summary of the histological findings in this group of cases is displayed in table 5.5.4.1. This table showed that CMV infection was present in 17 cases, 5 of them in Group IV and 12 in Group V (analysis for this finding will be described later). Lymphoma was seen in 8 cases, 3 of them from Group IV and 5 from Group V. These two opportunistic conditions were more common in Group V. Other neuropathological changes which were seen more frequently in Group V included peripheral nerve inflammation, which was present in 23 cases out of 27 (Peripheral nerve was not available in 2 cases of this group), mineralization and axonal balloons. The latter was present in 18 out of 29 cases of Group V and 21 cases of Group IV.

Table 5.5.4.1. Frequency of pathological changes in 78 AIDS cases

FINDINGS	PRESENT			
	GROUP IV N=49		GROUP V N=29	
	Number	%	Number	%
HIVE	31	**63.2**	13	**44.8**
PV Lymphocytic infiltrate	38	**77.5**	18	**62**
Microglial Nodules	40	**81.6**	22	**75.8**
Neuronophagia	20	**40.8**	11	**37.93**
Axonal Balloons	21	**42.8**	18	**62.06**
Vascular damage	10	**20.4**	4	**13.7**
Mineralization	11	**22.4**	9	**31**
CMV	5	**10.2**	12	**41.3**
Lymphoma	3	**6.1**	5	**17.2**
Peripheral nerve inflammation	29	**74.3**	23	**85.1**

All the remaining neuropathological changes investigated including HIVE, vascular damage, white matter damage, neuronophagia, perivascular inflammatory infiltrate and microglial nodules were more common in Group IV.

Table 5.5.4.2 shows the distribution of the results of neuropsychological evaluation by group. This table showed that neuropsychological evaluation was available in 46 cases of Group IV and 27 cases of Group V. A higher proportion of individuals in Group V, 59.2% (16 cases) were considered normal compared to 32.6% (15 cases) of Group IV.

Table 5.5.4.2 Distribution of Cognitive Impairment by Group

COGNITIVE IMPAIRMENT	IV		V		TOTAL
	N	%	N	%	
Not present	15	**32.6**	16	**59.2**	31
Mild	15	**32.6**	7	**25.9**	22
Moderate	2	**4.3**	1	**3.7**	3
Severe	14	**30.4**	3	**11.1**	17
Total	46	**100**	27	**100**	78

Data was missing in three cases of Group IV and two cases of Group V

For group IV, 31 (67.3%) cases out of 46 with neuropsychological evaluation were considered to have a degree of cognitive impairment ranging from mild to severe. In contrast, 11 cases (40.7%) out of 27 of group V were considered cognitively impaired.

This association between risk factor and dementia was significant (X^2= 5.248, $p<0.02$).

Drug users (Group IV) had a higher proportion of cognitively impaired individuals and in 14 of them (30.4%) the impairment was severe. In contrast, only 3 (11%) cases from Group V had severe cognitive impairment (table 5.5.4.2).

The histological finding which was strongly associated with cognitive impairment was HIVE. 73 cases from Groups IV and V had neuropsychological evaluation. Of these cases, 31 had normal cognitive function, 21 (67.7%) of whom had no evidence of HIVE at the histological level. 42 (57.5%) cases had a degree of cognitive impairment and 31 (73.8%) of them also had HIVE. This association was highly significant (X^2=14.319, $p<0.0005$). Analysis of groups individually showed that 26 cases with both conditions were from group IV. Thus, the association between

cognitive impairment and HIVE was only significant in the drug using group (X^2=12.983 p<0.0005).

There were 11 cases with cognitive impairment without histological evidence of HIVE. Analysis of those cases showed that 5 cases were from Group IV, accounting for 10.8% of all cases in that group and 6 were from Group V (22.2% of all Group V cases). Five cases had CMV infection at histological examination, 4 of them were in Group V. Two cases of Group V had lymphoma and one of these cases also had CMV infection. Axonal balloons were seen in 6 cases and all were in Group V. Microglial nodules were seen in 7 cases 4 of them of group V, and neuronophagia was seen in 4 cases, all of them in group V.

None of the comparisons between these histological features in the absence of HIVE and cognitive impairment reached significance in these cases even after analyzing each group separately.

Table 5.5.4.3 shows the distribution of cases with neuropsychological evaluation by ApoE genotype and group. This table showed that a higher proportion of cognitively intact individuals in both groups had a 3\3 ApoE genotype. For group IV, 11 cases out of 15 cognitively intact cases had a 3\3 ApoE genotype, 3 were 3\4 and 1 was 3\2. For Group V, 8 cognitively intact cases had 3\3 ApoE genotype, 2 were 3\4, 5 were 3\2 and one was 4\4. This table also shows that only 4 cases out of 20 with 3\3 ApoE genotype and cognitive impairment had severe dementia. Three of these cases were from Group IV and the other from Group V. For ApoE 3\4 cases, 4 out of 9

cases with cognitive impairment had severe dementia. In Group IV, 8 out of 9 cases with one or two ApoE *ε2* alleles were cognitively impaired and 7 had severe dementia. In Group V, however, only 3 out of 8 cases with one or two ApoE *ε2* alleles were cognitively impaired and in two of them the impairment was severe.

Table 5.5.4.3 Distribution of Neuropsychologically evaluated cases by ApoE genotype and Group.

GROUP	APOE GENOTYPE	COGNITIVE IMPAIRMENT							
		Not present		Mild		Moderate		Severe	
		No	%	No.	%	No	%	No	%
	3\3 n=25	11	**23.9**	9	**19.6**	2	**4.3**	3	**6.6**
Group IV	3\4 n=10	3	**6.6**	3	**6.6**	0	**0**	4	**8.7**
	3\2 n=6	1	**2.1**	0	**0**	0	**0**	5	**10.9**
N=46	2\2 n=2	0	**0**	1	**2.1**	0	**0**	1	**2.1**
	4\4 n=2	0	**0**	2	**4.3**	0	**0**	0	**0**
	2\4 n=1	0	**0**	0	**0**	0	**0**	1	**2.1**
	Total	15	**32.6**	15	**32.6**	2	**4.3**	14	**30.4**
	3\3 n=14	8	**29.7**	5	**18.5**	0	**0**	1	**3.7**
Group V	3\4 n=4	2	**7.4**	2	**7.4**	0	**0**	0	**0**
	3\2 n=8	5	**18.5**	0	**0**	1	**3.7**	2	**7.4**
N=27	4\4 n=1	1	**3.7**	0	**0**	0	**0**	0	**0**
	Total	16	**59.2**	7	**25.9**	1	**3.7**	3	**11.1**

Statistical analysis of the association between cognitive impairment and ApoE genotype in the 73 cases with neuropsychological evaluation confirmed a significant relationship. Of the 73 cases, 17 had 1 or 2 ApoE *ε2* alleles. Nine cases were in Group IV and 8 in Group V. Six cases had normal cognitive function and 11 had cognitive impairment. Of these 11 cases 9 had a severe cognitive impairment. The association between ApoE *ε2* allele and cognitive impairment was significant (X^2=7.095, p<0.008), but after analyzing groups separately it was only significant in Group IV (X^2=6.958, p<0.008).

Similarly, cognitive impairment was negatively associated with ApoE *ε3* allele. 39 cases of the 73 cases with neuropsychological evaluation had a 3/3 ApoE genotype. Of those, only 4 were considered to have a severe cognitive impairment. In contrast, 11 cases out of 28 with only one ApoE *ε3* allele were considered severely impaired. This negative association reached significance (X^2=5.104, p<0.02).

This negative association was only significant in Group IV (X^2=5.670, p<0.01).

Comparisons between cognitive impairment and ApoE *ε4* allele did not reach significance, although from table 5.5.4.3 it is evident that 12 cases out of 17 with one or two *ε4* alleles were cognitively impaired.

Another neuropathological change apart from HIVE significantly associated with cognitive impairment was neuronophagia.

Table 5.5.4.4 shows the distribution of cases with neuropsychological evaluation and neuronophagia by group.

Table 5.5.4.4 Distribution of cases with neuropsychological evaluation and neuronophagia by group.

GROUP	NEURONOPHAGIA	COGNITIVE IMPAIRMENT				
		Not present	Mild	Moderate	Severe	Total
	Negative	11	6	1	8	26
Group IV	Present	4	9	1	6	20
N=46	Total	15	15	2	14	46
	Negative	13	4	0	0	17
Group V	Present	3	3	1	3	10
N=27	Total	16	7	1	3	27

23 cases had neuronophagia and cognitive impairment, 16 of them were in Group IV and 7 in Group V. Analysis of table 5.5.4.4 shows that 15 out of 46 cases (32.6%) of Group IV had cognitive impairment without neuronophagia. In contrast, only 4 cases (14.8%) in Group V had mild cognitive impairment without histological evidence of neuronophagia. A significant association between neuronophagia and cognitive impairment was found (X^2=4.454, P<0.03). This association was only present in Group V (X^2=8.541, p<0.003). No significant associations were found between neuronophagia and ApoE genotype.

HIVE

The association between HIVE and cognitive impairment was shown previously.

Table 5.5.4.5 shows the distribution of cases with HIVE by Group.

Table 5.5.4.5 Distribution of cases with HIVE by group.

GROUP	HIVE							
	Negative		Mild		Florid		Total	
	No.	%	No.	%	No.	%	No.	%
Group IV N=49	18	**36.7**	9	**18.3**	22	**45.8**	49	**100**
Group V N=29	16	**55.1**	7	**24.1**	6	**20.8**	29	**100**

Analysis of table 5.5.4.5, showed that in 34 cases there was no histological evidence of HIVE. 18 of these cases (36.7%) were in Group IV and 16 (55.2%) in Group V. 31 cases of Group IV (63.3%) and 13 cases of Group V (44.8%) had histological evidence of HIVE. The proportion of cases with florid HIVE was higher for group

IV (45%) than for Group V (20.8%). Cross-tabulation of risk group and HIVE showed a significant association between these two variables (X^2= 4.146, $p<0.05$). Table 5.5.4.6 shows the distribution of cases with HIVE by ApoE genotype and group.

Table 5.5.4.6 Distribution of cases with HIVE by ApoE genotype and group

GROUP	APOE GENOTYPE	HIVE							
		Negative		Mild		Florid		Total	
		No	%	No	%	No	%	No	%
Group	3\3	14	**28.5**	5	**10.2**	8	**16.3**	27	**55**
IV	3\4	4	**8.1**	3	**6.1**	4	**8.1**	11	**22.7**
	3\2	0	**0**	0	**0**	6	**12.2**	6	**12.2**
N= 49	2\2	0	**0**	0	**0**	2	**4.0**	2	**4.0**
	4\4	0	**0**	0	**0**	2	**4.0**	2	**4.0**
	2\4	0	**0**	1	**2.0**	0	**0**	1	**2.0**
	Total	18	**36.6**	9	**18.3**	22	**44.8**	49	**99.9**
	3\3	9	**31.0**	3	**10.3**	3	**10.3**	15	**51.7**
Group V	3\4	2	**6.9**	2	**6.9**	1	**3.4**	5	**17.2**
	3\2	4	**13.8**	2	**6.9**	2	**6.9**	8	**27.6**
N=29	4\4	1	**3.4**	0	**0**	0	**0**	1	**3.4**
	Total	16	**55.1**	7	**24.1**	6	**20.6**	29	**99.9**

Analysis of table 5.5.4.6 showed that most cases with ApoE 3/3 genotype in both groups had no evidence of HIVE at microscopical examination. In contrast, cases with an ApoE genotype different from 3\3 tend to have more severe HIVE. This was evident only in Group IV and specifically in cases with ApoE *ε2* allele. For example, all 9 cases with one or two ApoE *ε2* allele in Group IV had evidence of HIVE, and in 8, it was florid. Thus, of 34 cases without HIVE, 23 (67.6%) of them were 3/3, and 14 of these were in Group IV. In contrast, 6 cases out of 15 (30%) with 3/4 genotype and 4 cases out of 14 (28.6%) with 3/2 genotype had no evidence of HIVE.

In Group IV a significant association was found when ApoE genotype (all six) were cross-tabulated against HIVE (X^2=20.453, p<0.02). When individual alleles were cross-tabulated against HIVE, significant associations were found for ApoE *ε2* (X^2=7.999,p<0.005) and a negative association of HIVE with ApoE *ε3* (X^2=7.610, p<0.006). The *ε4* allele was not found to be associated with HIVE.

Neuronophagia was significantly associated with HIVE (X^2=8.682, p<0.003).

Analysis of table 5.5.4.7 showed that in group IV, 34.7% (17 cases) and 31% (9 cases) in group V had HIVE but neuronophagia was not seen in any of the brain areas studied. 38.8% (19 cases) of Group IV had both conditions and only 13.7% (4 cases) on Group V had both conditions. After analyzing groups separately, neuronophagia and HIVE were associated only in the drug-using group, Group IV, (X^2= 13.208, p<0.0005).

Table 5.5.4.7 shows the distribution of cases with neuronophagia and HIVE by group.

Table 5.5.4.7 Distribution of cases with HIVE and neuronophagia by group

GROUP	NEURONOPHAGIA	HIVE							
		Negative		Mild		Florid		Total	
		No.	%	No.	%	No.	%	No.	%
Group IV N=49	Absent	17	**34.7**	4	**8.1**	8	**16.3**	29	**59.2**
	Present	1	**2.0**	5	**10.2**	14	**28.6**	20	**40.8**
Group V N=29	Absent	9	**31.0**	6	**20.7**	3	**10.3**	18	**62.0**
	Present	7	**24.1**	1	**3.4**	3	**10.3**	11	**38.0**

Neuronophagia was not associated with ApoE genotype as previously mentioned.

Table 5.5.4.8 shows the distribution of cases with HIVE and perivascular lymphocytic inflammatory infiltrate, by group.

Table 5.5.4.8 Distribution of cases with HIVE and perivascular inflammatory infiltrate by group

GROUP	PERIVASCULAR LYMPHOCYTIC INFILTRATE	HIVE							
		Negative		Mild		Florid		Total	
		No.	%	No.	%	No.	%	No.	%
Group IV N=49	Absent	2	**4.0**	0	**0**	9	**28.4**	11	**22.4**
	Present	16	**32.6**	9	**18.4**	13	**26.6**	38	**77.6**
	Total	18	**36.6**	9	**18.4**	22	**45.0**	49	**100**
Group V N=29	Absent	4	**13.8**	3	**10.3**	4	**13.8**	11	**38**
	Present	12	**41.4**	4	**13.8**	2	**6.9**	18	**62**
	Total	16	**65.2**	7	**24.1**	6	**20.7**	29	**100**

Analysis of table 5.5.4.8 showed that of 34 cases without HIVE, 28 (82.4%) had perivascular lymphocytic inflammatory infiltrate. For Group V 12 cases out of 18 (66.6%) without HIVE, had perivascular lymphocytic inflammatory infiltrate. In contrast, 6 cases of that group had both conditions.

HIVE was negatively associated with perivascular lymphocytic inflammatory infiltrate (X^2=5.985, $p<0.01$).

Microglial nodules were present in 62 cases (40 of Group IV and 22 of Group V). 43 of these 62 cases had also HIVE and 28 of them had florid HIVE. Only one case, in which occasional microglial nodules were seen, had no histological evidence of HIVE. Microglial nodules were seen in 19 cases without HIVE. A highly significant association between microglial nodules and HIVE was found (X^2= 18.653, $p<0.0005$). Microglial nodules were seen in 15 cases out of 17 with CMV infection,

but no significant association between these two conditions was found, probably due to the high frequency of microglial nodules in HIVE cases.

Table 5.5.4.9 shows the distribution of cases with HIVE and axonal balloons by group.

Table 5.5.4.9 Distribution of cases with HIVE and axonal balloons by group

GROUP	AXONAL BALLOONS	HIVE							
		Negative		Mild		Florid		Total	
		No.	%	No.	%	No.	%	No.	%
	Negative	15	**30.6**	5	**10.2**	8	**16.3**	28	**57.1**
Group IV	Positive	3	**6.1**	4	**8.1**	14	**28.6**	21	**42.9**
N=49	Total	18	**36.7**	9	**18.3**	22	**44.9**	49	**100**
	Negative	5	**17.2**	2	**6.9**	4	**13.8**	11	**37.9**
Group V	Positive	11	**37.9**	5	**17.2**	2	**6.9**	18	**62.0**
N=29	Total	16	**55.1**	7	**24.1**	6	**20.7**	29	**99.9**

Analysis of table 5.5.4.9 showed that axonal ballons were present in 39 cases, 21 were in Group IV and 18 in Group V. In Group IV, HIVE was seen in 31 cases, and 22 had florid HIVE. 14 cases with florid HIVE (63.6%) had axonal ballons. In contrast, for Group V, florid HIVE was present in 6 cases and 2 (33.3%) also had axonal balloons. For Group IV, 18 cases had no HIVE, and axonal balloons were seen in 3 cases (16.6%). In contrast in 16 cases of Group V, HIVE was not seen histologically, and 11 of these cases (68.7%) had axonal balloons. Axonal balloons were also found associated with HIVE (X^2=8.693, $p<0.003$), but only in Group IV

Cytomegalovirus infection (CMV).

Histological evidence of CMV infection was present in 17 cases, 12 of which were from Group V. Significant association between risk group and CMV infection was

found (X^2=10.255, p<0.001). All 17 cases had less than 22 CD4+T cell counts. Thus, CMV infection was significantly associated with CD4 counts (X^2= 9.425, p<0.002).

None of the other histological findings were associated with dementia, HIVE or risk factor.

5.5.5. Relation of Neuropathological findings with CD4 and CD8 counts

Table 5.5.5.1 shows the distribution of cases with perivascular lymphocytic infiltrate by quartiles of CD8 counts.

Table 5.5.5.1 Distribution of cases with perivascular lymphocytic infiltrate by quartiles of CD8 counts.

PERIVASCULAR MONONUCLEAR INFILTRATE	CD8 QUARTILES				
	1	2	3	4	Total
Negative	8	6	6	1	21
Positive	8	15	13	18	54
Total	16	21	19	19	75*

*CD8 counts were missing in 3 cases.

Analysis of table 5.5.5.1 showed that almost all cases (18 out of 19) with CD8 counts in the 4th quartile had perivascular lymphocytic inflammatory infiltrate and 13 out of 19 cases in the 3rd quartile also did. In contrast, cases in the first quartile were equally distributed between having or not having mononuclear inflammatory infiltrate around blood vessels. This association between perivascular inflammatory cells and CD8 counts was significant (X^2=7.281, p<0.007). The association between perivascular infiltrate and CD4 counts also reached significance (X^2=4.418, p<0.03).

A negative significant association between CD8 cell counts and HIVE was found in Group IV. Most of the cases with HIVE (19 out of 31) had CD8 cell counts below the 50th percentile (X^2=4.639, p<0.03). The opposite was found for CD4 counts. Most of the cases with HIVE (19 out of 31) had CD4 cell counts above the 50th percentile but this failed to reach statistical significance.

5.5.6 ApoE Genotype and Viral Conditions

The frequency of ApoE genotypes in the 78 cases studied and in cases with evidence of HIVE, CMV infection and lymphoma in the brain are displayed in table 5.5.6.1. The frequency of individual alleles is shown in table 5.5.6.2.

Table 5.5.6.1 Frequency of ApoE genotypes in the AIDS population, (Groups IV and V) and cases with productive viral infections of the brain

APOE GENOTYPE	STUDY POPULATION		HIVE		CMV		LYMPHOMA	
	No.	%	No.	%	No.	%	No.	%
3\3	42	**53.8**	19	**43.2**	8	**47.1**	3	**37.5**
3\4	16	**20.5**	10	**22.7**	4	**23.5**	1	**12.5**
3\2	14	**17.9**	10	**22.7**	4	**23.5**	4	**50**
2\2	2	**2.6**	2	**4.5**	1	**5.9**	0	**0**
4\4	3	**3.8**	2	**4.5**	0	**0**	0	**0**
2\4	1	**1.3**	1	**2.2**	0	**0**	0	**0**
Total	78	**99.9**	44	**99.9**	17	**100**	8	**100**

Table 5.5.6.2 Frequency of ApoE alleles in the study population and cases with viral infection of the brain.

APOE ALLELES	STUDY POPULATION		HIVE		CMV		LYMPHOMA	
	No.	%	No.	%	No.	%	No.	%
ε2	19	**12.1**	15	**17**	6	**17.6**	4	**25**
ε3	114	**73**	58	**65.9**	24	**70.5**	11	**68.7**
ε4	23	**14.9**	15	**17**	4	**11.7**	1	**6.2**
Total	156	**100**	88	**99.9**	34	**99.9**	16	**99.9**

Analysis of tables 5.5.6.1 and 5.5.6.2 showed that the *ε2* allele of ApoE was over represented in all cases with viral infections of the brain when compared with the study population. Comparisons among these populations did not reach significance. Although few cases had primary lymphoma of the brain, 25% of the cases had the 3\2 ApoE genotype but the association of ApoE genotype and lymphoma in the studied population did not reach significance. Nevertheless, the frequency of the ApoE *ε2* allele in these viral conditions was even higher than the frequency of the same allele in the drug user cohort in which ApoE *ε2* is over-represented (table 3.2.2). When these frequencies are compared with published frequencies of individual alleles in a cohort of normal Scottish population (Group VI in table 3.2.2), the difference is even higher.

5.6. Microglial/Macrophage quantitation (CD68)

Immunohistological stains for CD68 disclosed morphologically small, elongated bipolar cells without evidence of ramification in both the grey and white matter of control cases (Group I). In HIV negative and HIV positive drug users (Group II and

III), CD68 positive cells were enlarged and showed cytoplamic processes, frequently ramified. Occasionally, they were seen in small clusters forming microglial nodules. These cells were seen in both white and grey matter. In AIDS cases, the increased size of the CD68 positive cells was evident. The cytoplamic processes had well defined ramifications. Microglial nodules were larger than those observed in Groups II and III, and this feature was also seen in both white and grey matter. Interestingly, in the grey matter CD68 positive cells were frequently seen around neurons. In AIDS cases macrophages and multinucleated giant cells were also positive for CD68.

Immunohistological stains for macrophages/microglial cells were carried out in histological sections of posterior frontal, temporal and thalamus of 36 cases. Cases were divided into 5 groups, Group I included normal controls (n=4), Group II, HIV negative drug users (n=10), Group III, pre-symptomatic HIV positive drug users (n=7), Group IV, HIVE drug users (n=9) and Group V, HIVE non-drug users (n=6). This is illustrated in table 5.6.1

Table 5.6.1 Distribution of cases studied for CD68 by ApoE genotype and Group.

APOE GENOTYPE	3\3	3\4	3\2	2\2	4\4	2\4	TOTAL
Group I N=4	3	1	0	0	0	0	4
Group II N=10	6	3	0	0	1	0	10
Group III N=7	5	2	0	0	0	0	7
Group IV N=9	3	2	3	0	0	1	9
Group V N=6	3	1	2	0	0	0	6

Nine consecutive 20x microscopical fields were analyzed separately for grey and white matter of each section using a Leica Q500iw system (Fig. 5.5). The program

used for quantification expressed the results as total positive pixels for CD68 immunostained sections (Fig. 5.6). This program has been standardized at the Creutzfeldt–Jakob disease (CJD) Unit of the Western General Hospital (Edinburgh) (Courtesy of Dr. W. Nailon)

The mean values for total number of positive pixels for CD68 detected in all the nine fields of grey matter and separately for white matter of each anatomical area were used for comparisons of homologous areas of different groups (Data displayed in Appendix IV).

Analysis of the results was carried out using analysis of variance (ANOVA) after normalization of the data by natural logarithmic transformation of the values. Tukey HSD post-Hoc tests were used for comparing individual groups.

Differences in mean and median values for grey and white matter among the three anatomical areas studied in each group were observed. All white matter areas had higher values than grey matter areas with the exception of higher values in the grey matter of the thalamus in Group IV.

Figure 5.5 Microglial quantitation

Microglial quantitation was carried out using a Leica Q 500iw image analysis system. Black and white images of CD68 immunostained sections were analyzed.

Grey matter of temporal hippocampus of an AIDS case with an ApoE 3\3 phenotype (a). (DAB with haematoxylin counterstain X200).

Grey matter of temporal hippocampus of an AIDS case with an ApoE 3\2 genotype (b). (DAB with haematoxylin counter stain X200).

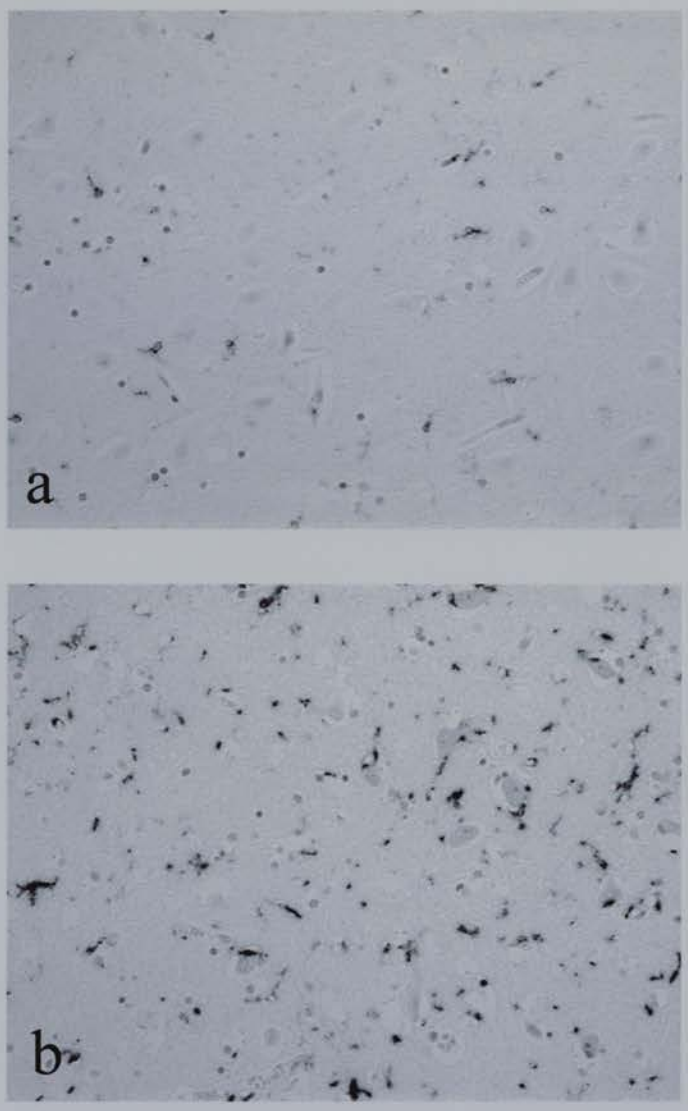

Figure 5.5

Figure 5.6 CD68 Immunostaining

CD68 Immunostaining of the dentate fascia of the temporal hippocampus of an ApoE 3\3 AIDS case (DAB with haematoxilin counterstain X200).

The same anatomical area of an ApoE 3\2 AIDS case (DABwith haematoxilin counterstain X200). Higher number of CD68 positive cells was observed in AIDS cases with an ApoE genotype different from 3\3.

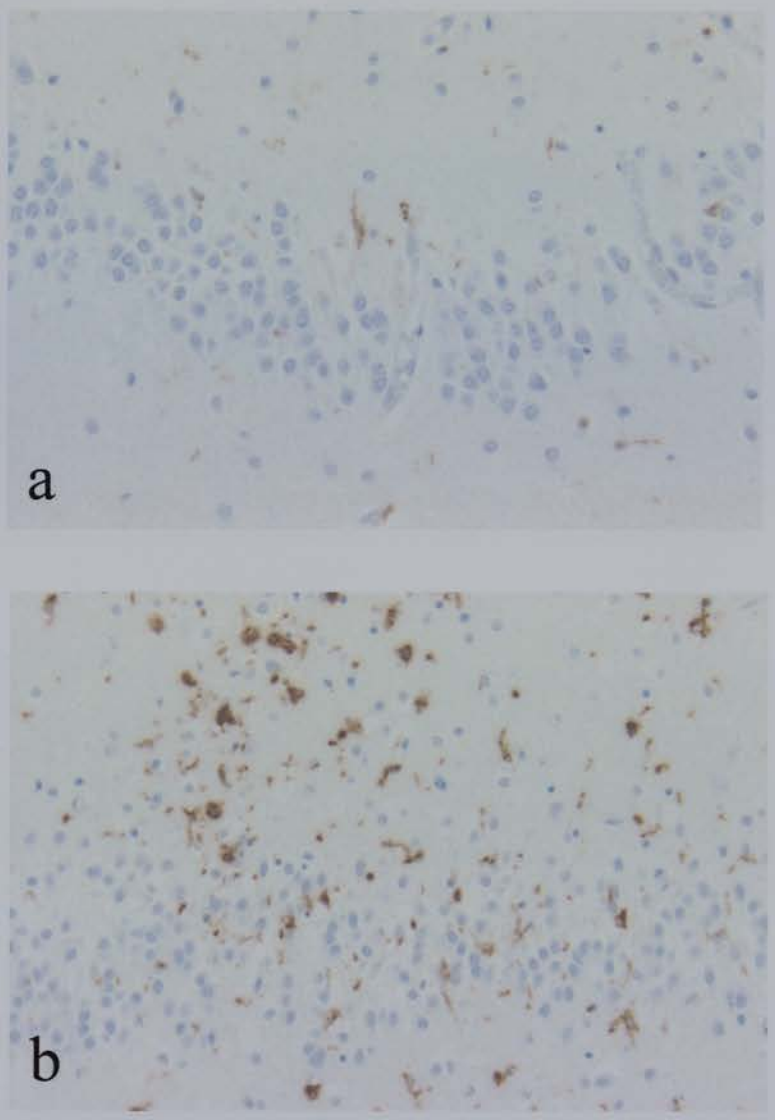

Figure 5.6

For the HIV negative groups (Groups I and II), the highest median value for all grey matter studied was observed in the temporal hippocampus section. It was followed by the frontal cortex in Group I and the thalamus in Group II.

For the HIV positive groups (Groups III, IV, V), the highest median values for grey matter areas were obtained in the frontal cortex for Groups III and V, followed by the thalamus. For Group IV, the highest median value was observed in the thalamus followed by the temporal hippocampus.

For white matter areas, Group I had the highest median value in the temporal hippocampus followed by the frontal lobe, similar to the observations for grey matter areas. For Groups II and III, (both drug using groups) however, the highest median value for white matter areas was obtained in the frontal lobe section followed by the temporal hippocampus, while in Group IV it was in the temporal hippocampus followed by the frontal lobe. For Group V almost equal values were obtained in frontal and temporal sections.

In summary, almost all white matter values were higher than grey matter values for each anatomical section in all five groups studied, with the exception of the grey matter of the thalamus in Group IV, which had slightly higher values that those of the white matter. In Group IV, higher values for grey matter were obtained in the thalamus, and in the white matter of the temporal hippocampus, than in any other area, which made this group different from the other HIV positive groups (III and V).

When comparisons between the groups were carried out, no significant differences were observed between Groups I and II and III in any of the six areas studied, although generally increasing values were observed in Group II compared with

Group I and in Group III compared with Group II. When each of groups I and II were compared with Groups IV and V, significant and highly significant differences were obtained in all six areas studied.

Group III did not differ significantly from Group V in any of the white matter areas. There were significant differences between Groups III and IV for mean values in the white matter of thalamus ($p<0.02$), but not in any other white matter area. Comparisons of grey matter areas between Groups III and IV showed significant differences in the thalamus ($p<0.004$) and temporal hippocampus ($p<0.0005$). Group III also differed significantly from Group V in mean values obtained in the grey matter of temporal lobe ($p<0.004$).

None of the comparisons between Groups IV and V were significant.

In summary, HIV positive drug using groups (Groups III and IV) were significantly different in the positivity for CD68 in the grey matter areas of the temporal hippocampus and thalamus and in the white matter of the thalamus. Pre-symptomatic HIV positive cases and HIVE non-drug users only differed in the grey matter of the temporal lobe.

5.6.1 CD68 and ApoE Genotype

The ApoE genotype of the 4 cases in Group I was 3\3 for 3 cases and 3\4 for one. Six cases from Group II were 3\3, 3 were 3\4 and one was 4\4. From Group III, 5 cases were 3\3 and 2 cases were 3\4. From Group IV, 3 cases were 3\3, 3 were 3\2, 2 were 3\4 and one case was 2\4. For Group V, 3 cases were 3\3, 2 were 3\2 and one was 3\4 (Table 5.6.1).

In the frontal grey matter, all 3\3 cases of all groups studied had lower mean and median values for the total number of positive pixels for CD68. Differences were more evident for Groups IV and V. In Group IV, the mean and median values for 3 cases with 3\3 ApoE genotype were 921.2 and 1047.6 respectively, while for 3 cases in the same group with 3\2 ApoE genotype the values were 2363.8 and 2378.4 respectively. This was also observed in all other grey matter areas and in all white matter areas, but only in Group IV and V cases.

Since there were no significant differences in the total number of positive CD68 cells between Groups IV and V, these two groups were analyzed together when carrying out comparisons between ApoE genotypes. The total number of cases analyzed was 15. All six areas were analyzed independently and the values for total number of positive pixels were categorized in quartiles. The ApoE genotype of these cases was: 3\3 for 6 cases, 3\4 for 3 cases, 3\2 for 5 cases and 2\4 for one case.

Significant associations between ApoE genotype and total positivity in pixels for CD68 were obtained in the grey and white matter of the temporal lobe and in the grey matter of the frontal lobe in these AIDS cases. In the temporal grey matter, the mean value for the first quartile was 755.2 and there were 3 cases in this quartile. The second quartile included 4 cases with a mean value of 1297.4. The third and fourth quartiles included 4 cases each and the mean values were 1752.3 and 3820.3 respectively.

Regarding ApoE genotype, mean value for CD68 positivity in cases with 3\3 ApoE genotype was 1073.6. For these cases, the minimum value was 195.2 and the maximum value was 1760. These cases were distributed into quartiles as follows: 3 cases were in the first quartile (all the cases in that quartile), 2 cases were in the

second quartile, one case in the third quartile and no cases in the fourth quartile. For cases with 3\4 ApoE genotype, the mean value was 3195.8 and all cases with this genotype were in the fourth quartile. For cases with 3\2 ApoE genotype, the mean value was 2510.6. For these cases the minimum value was 1609.7 and one case was in the second and fourth quartiles and 3 cases were in the third quartile.

There was only one case with 2\4 ApoE genotype with a mean value for total CD68 positivity was 1164.3 and it was in the second quartile.

These four different types of ApoE genotype were cross-tabulated against total positivity for CD68 cells. Results of these cross-tabulations disclosed a significant association (X^2=18.875, $p<0.02$). The significance increased when cases were grouped by having or not having a 3\3 ApoE genotype. Thus, 6 cases with 3\3 ApoE genotype were compared with 9 cases with non 3\3 ApoE genotypes (X^2=6.929, $p<0.008$). These findings also applied for white matter areas of the temporal lobe (X^2=6929, $p<0.008$).

Regarding the gray matter of the frontal lobe, the mean values for total CD68 positivity for the first quartile was 744.7 with 3 cases included in it. All other quartiles (second, third and fourth) included 4 cases each and the mean values were 1321.3, 1905.4 and 3103.1 respectively.

Mean value for total positivity in pixels for CD68 cells for cases with ApoE genotype 3\3 was 1345.6. Minimum and maximum values for these cases were 170.2 and 2798 respectively. Three cases were in the first quartile, two in the second quartile and one in the fourth. For cases with a 3\4 ApoE genotype, the mean value was 2101.2, and there was one case in the second, third and fourth quartiles. For cases with a 3\2 ApoE genotype, the mean value was 2284.8 with one case in the

second quartile and two cases in the third and fourth quartiles. The case with a 2\4 ApoE genotype was in the second quartile and its mean value was 1751.8 positive pixels.

When cases with and without 3\3 ApoE genotype were cross-tabulated against total CD68 cells positivity, the association was again significant ($X^2=4.676$, $p<0.03$).

In summary, cases with *ε3* were associated with the lowest and cases with *ε4* were associated with the highest CD68 counts with *ε2* cases having intermediate values.

Chapter 6

DISCUSSION

After a brief summary of the main findings, the discussion of the results will be presented in the following order:

Discussion of the findings regarding the frequency of ApoE alleles among the different groups of cases studied.

Discussion of the findings regarding the differences in CD4 and CD8 T cell counts among cases with: a) different risk factor for contracting HIV infection and b) different ApoE genotypes.

Discussion of the effects of drugs of abuse.

Discussion of differences in the neuropathological findings among risk groups and in relation to ApoE genotype.

Discussion of the findings on microglial quantitation among risk groups and ApoE genotype.

The main results of the present study may be summarized as follows:

The frequency of ApoE $\varepsilon 2$ allele in a cohort of HIV positive drug users was significantly higher than the frequency of the same allele in a group of HIV negative drug users, normal DNA donors and the published frequency of that allele in the normal Scottish population.

Analysis also showed significant differences in the last CD4+ T cell counts and CD4/CD8 ratio between drug users and non-drug users with AIDS. A significant

association between CD8+ T cell counts and ApoE genotype was found for the whole cohort of cases and in particular for drug users with AIDS. The ApoE *ε2* allele was significantly associated with higher values for the last CD8+ T cell counts in both groups while the *ε4* allele was negatively associated with CD8+ T cell counts in the non-drug users group. The last CD8+ T cell counts was significantly associated with the presence of perivascular lymphoid infiltrate at histological examination and it was negatively associated with histological evidence of HIVE.

In the drug-using group, HIVE was more frequently observed than in non-drug users, while opportunistic conditions were seen more frequently in the latter group. In drug users, a highly significant association between HIVE and cognitive impairment was also observed. A highly significant association was also found between HIVE and *ε2* allele of the ApoE and negatively associated with the *ε3* allele of the ApoE.

Although non-significant, a larger number of CD68 positive macrophage/microglial cells was found in the HIV negative drug-using group than in normal controls. The number of CD68 positive cells was found significantly higher in drug users and non-drug users with AIDS than in HIV negative groups. An intermediate number of CD68 positive cells were found in the HIV positive pre-symptomatic drug-using group. Significant differences between this group and HIV positive drug users with AIDS were found in the grey matter of the temporal hippocampus and thalamus and white matter of the thalamus. The non-drug user group with AIDS differed significantly from pre-symptomatic cases in the number of positive CD68 cells in the grey matter of the temporal hippocampus.

Finally, when comparisons between ApoE genotypes and CD68 positive cell counts were carried out in the two AIDS groups (drug users and non-drug users) analyzed

together, significantly lower values were found for cases with ApoE 3\3 genotype than for cases with all other genotypes in the grey and white matter of the temporal hippocampus and grey matter of the frontal lobe.

6.1. Frequency of ApoE alleles

The high frequency of the ApoE *ε2* allele among HIV positive drug users (14%) raises the possibility that possession of this allele and the simultaneous use of drugs may increase the susceptibility to contract HIV infection. The finding that the *ε2* was not over-represented in HIV positive non-drug users, shows that *ε2* on its own does increase susceptibility to contract HIV infection. In the HIV positive cohort of drug users, a higher frequency of some ApoE genotypes such as 2\2 and 2\4 was found when compared to the normal Scottish population (4.1% vs. 0.5% and 5.7% vs. 2.8% respectively). These two genotypes are found in Caucasians in a frequency of approximately 1-2% (Mahley and Rall, 2000). In the HIV Positive non-drug users, none of the cases had these so-called "rare" genotypes as may be expected perhaps for a population sample of 44 Caucasian cases.

In recent years it has become clear that ApoE genotype and specific ApoE alleles have a direct influence in different systemic diseases such as atherosclerosis and in some neurodegenerative diseases such as Alzheimer's disease.

It is now recognized that the ApoE *ε4* has been linked to increased risk of coronary artery disease (Cumming and Robertson, 1984).

The *ε4* allele has been shown to be an important risk factor for the development of sporadic and late onset familial Alzheimer's disease (Strittmatter et al, 1993; Saunders

et al, 1993). The risk for developing Alzheimer's disease was found to be gene dose dependent (Corder et al, 1993). In other neurodegenerative diseases such as Parkinson's disease, there is conflicting evidence. An over-representation of the *ε2* has been reported among familial Parkinson's disease cases (FPDGSG, 1997), but the ε4 allele has also been shown to be over-represented in another study (Kruger et al, 1999). In multiple Sclerosis (MS), recent studies suggested defective remyelination in cases with the *ε2* allele (Carlin, 2000) while other studies have reported an association between the ApoE *ε4* allele with more aggressive MS cases (Evangelau et al, 1999) and also with impaired recovery after relapsing episodes of MS (Chapman et al, 1999). A high frequency of the ApoE *ε2* has also been reported in cases with cerebral amyloid angiopathy and this allele was significantly associated with haemorrhage in this condition (Nicoll et al, 1997). Significantly higher frequencies of the ApoE *ε2* allele have also been reported in argyrophilic grain disease (Ghebremedhin et al, 1998). The ApoE *ε4* has been associated with poorer outcome after head injury (Graham et al, 1999). In age-related macular degeneration, the most common cause of blindness in the elderly population in the Western World, a significant association was found between ApoE genotype and risk of having this condition. The ApoE *ε4* was associated with a protective effect while the ApoE *ε2* was associated with increased risk for age related macular degeneration (Klaver et al, 1998; Souied et al, 1998).

After taking all the above mentioned studies into consideration, it seems clear that ApoE genotype plays an important role in modulating both systemic diseases and neurological conditions.

In viral conditions, studies have shown an over-representation of ApoE *ε4* allele (and consequently under-representation of the *ε3* allele), in Herpes Simplex virus (HSV)

type 1 infection (Itzhaki et al, 1997). In that study, the frequency of ApoE *ε4* allele in herpes labialis sufferers was found to be 36.3%, while the frequency of ApoE *ε3* allele was 55%. Interestingly, the frequency for 2\4 ApoE genotype in that same study was 12.5% (the expected frequency of ApoE genotype 2\4 in Caucasian population is lower than 1%). It has been recently shown that the ApoE *ε2* allele is a risk factor for contracting HSV encephalitis (Lin et al, 2001). A high frequency of this ApoE allele (26%) was found in a cohort of HSV encephalitis cases. The proposed mechanism for this finding was specific interactions between HSV with heparan sulphate proteoglycan (HSPG) and a receptor of the low-density lipoprotein (LDL) receptor family. ApoE uses the same receptors to gain access to the cells, therefore competition of ApoE (which is isoform specific) with HSV for these receptors appears to mediate the increased risk for developing HSV encephalitis. Because HSV and HIV bind HSPG on the cell surface (reviewed in Mahley and Rall, 2000), a similar mechanism may be involved in the increased susceptibility of drug users for the initial HIV infection. It is of interest however, that this "susceptibility" is not observed among non-drug users. In Lin's paper HSV neurovirulent variants were also suggested to be involved in the increased risk of HSV encephalitis among ApoE *ε2* carriers. This can also apply to the present study because the drug-using cohort in Edinburgh is closely related (socially and geographically) and the possibility of a neurovirulent HIV variant among this population is highly probable given the point infection source of the outbreak in late 1983/early 1984 (Brettle et al, 1996).

Two other studies have compared ApoE genotypes and alleles among HIV positive patients and AIDS cases, but these studies have focused on the association of ApoE with HIV associated dementia. The ApoE *ε4* allele was investigated in 44 pre-AIDS

cases in one study (Corder et al, 1998). In this study, an excess of cognitive impairment was shown in 11 *ε4* carriers compared to non-*ε4* carriers. The frequency of the other ApoE alleles was not published. In the other study 132 post-mortem mixed risk group of AIDS cases were genotyped for ApoE and the frequency of all six genotypes and three alleles was reported not to be different from the normal Norwegian population (Dunlop et al, 1997). Some methodological differences between the last study and the present study may account for the difference in results. In the present study, a high frequency of ApoE *ε2* allele among 122 HIV positive drug users was found and the frequency of the same allele among HIV positive non-drug users was not significantly different from the published frequency for the normal Scottish population (Cumming and Robertson, 1984). In Dunlop's study, the selection criterion paid no account of risk group and was based purely on the availability of tissue for DNA extraction and all cases with a post-mortem examination were included. The exact proportion of the major risk factors for contracting HIV infection was not mentioned.

Another possible explanation for the ApoE genotype frequencies found in the present study, is that the drug using community in Edinburgh is closely related and members of the same family can be found among the HIV positive drug users group (Ann Chiswick personal communication). This possibility is exciting, because it may imply that other non presently identified gene(s) can be modulating HIV infection and HIV related disease progression independently of ApoE genotype or acting together with ApoE as has been postulated for other neurodegenerative conditions such as Parkinson's disease (Kruger et al, 1999). The high frequency of HIVE among Edinburgh's AIDS drug users (Bell et al, 1996, 1998) may also be explained by this possibility, but a more

comprehensive genetic survey of this population is required in order to address this issue.

6.2 CD4/CD8 T cell counts

Results also showed that the last recorded CD4 cell counts before death as well as the CD4/CD8 ratio differed significantly between the two main risk groups studied, drug users and non-drug users with AIDS. Increased values for CD4 cell counts were found in the drug-using group. A significant association was found between CD8 cell counts and ApoE genotype in the whole cohort of cases studied and in the drug-user group in particular (drug users had higher mean values for CD8 T cell counts). An opposite effect of ApoE *ε2* and *ε4* was found in respect of CD8 T cell counts. The *ε2* allele was associated with significantly higher values for the last CD8 count in the whole cohort. The *ε4* allele was found negatively associated with the last CD8 count in non-drug users group, thus *ε4* carriers had lower values for the last CD8 counts. A relationship between ApoE and CD4+ T cell counts was reported previously by Corder who reported that MSM ApoE *ε4* carriers tend to have fewer CD4+ T cells than non-*ε4* carriers (Corder et al, 1998). The mean CD4 lymphocyte counts for the patients included in that study was 350 cells/μl and for *ε4* carriers was 204 cells/μl. The present study of the CD4/CD8 relationship with ApoE status examined exclusively cases who had less than 200 CD4+ T cells. 10 of the non-drug user cases had one (8 cases) or two (2 cases) ApoE *ε4* allele(s), and none of the 10 had more than 80 CD4+ T cells/μl and only two had counts between 18-80 cells/μl (Table 3.3.6). This finding although non-significant, suggests that the effect of specific ApoE alleles on CD4 cells is not

restricted to AIDS cases since it was also observed in Corder's study of cases with more than 200 CD4+ T cells/μl.

These findings suggest that both risk groups might have a differential T cell homeostasis based on the individual ApoE genotype. One of the differences between cases of these two risk groups is age. The drug-using group was on average 5 years younger than the non-drug using group, but it is unlikely that the age differences between the groups is contributing to the difference in CD4 and CD4/CD8 ratio found between them since the significance of the association did not change after adjusting for age.

The homeostasis of CD4 and CD8 T cells is inter-related. HIV specific CD8+ T cells have been shown in peripheral circulation at high frequency even in the setting of low CD4 +T cell counts (Spiegel et al, 2000; Gea-Banacloche et al, 2000). In cases progressing to AIDS, loss of these HIV specific CD8 cells coincides with a dramatic loss of CD4 cells (Klein et al, 1995). It could be speculated that the higher counts of CD4+ T cells observed in AIDS drug users are reflected in high CD8 counts and CD8 function. The opposite is also true, the function of HIV specific CD8+ T cells also depends on the number and functional state of CD4+ T cells and more specifically on the cytokine milieu. Nevertheless, indirect evidence supporting the presence of active CD8 cells in AIDS drug users of the Edinburgh cohort exists. Even in cases with a low CD4 count and correspondingly low CD8 count, this study suggests that ApoE genotype governs the effectiveness of the residual immune response. Opportunistic conditions of the CNS were found in 63% of non-drug users cases but in only 31 % of the AIDS drug users (Bell et al, 1996), suggesting a degree of immune response in AIDS drug users.

In the present study, non-drug user cases had 2.4 fold more CMV infection than drug users, suggesting that a degree of CD8+ T cell function was present in the latter group, even in cases with low CD4 counts. Thus, equal numbers of cases (30) in both groups had fewer than 18 CD4+ T cells/μl, but 12 of the non-drug users had CMV infection compared to only 5 of the drug using group.

Results also indicate an effect of individual ApoE alleles on the homeostasis of T cells. Cases homozygous for the ApoE *ε3* allele had lower values for the last CD4 and CD8 cell counts when compared to individuals heterozygous for that allele (3\4 and 3\2 cases). Comparisons between homozygous cases (3\3, 2\2 and 4\4) are of limited value due to the relative small frequency of cases homozygous for ApoE *ε2* and *ε4* alleles. When serum lipid levels among individual ApoE genotypes have been compared in previous studies (Dallongeville et al, 1992, Grunfeld 1997) it is assumed that the normal parameter with which comparisons are carried out is set by ApoE 3\3 genotype. When the same approach is adopted for T cell counts, independently from the risk factor, cases heterozygous for *ε3* (3\4 and 3\2) have higher mean and median values for CD4 and CD8 counts than those homozygous for *ε3*. Cases with 3\2 genotype have higher mean and median values for both CD4 and CD8 counts than cases with 3\4 genotype and these latter (3\4) cases have higher values for both measurements than 3\3 cases, although the difference is not as marked. Thus, the effect of different ApoE alleles with respect to T cell counts is similar to the effect of different ApoE alleles on serum lipid levels. Although the number of cases in the present study is limited, it is tempting to speculate that levels of lipids might be influencing (by an unknown mechanism) the homeostasis of T cells in AIDS cases.

In AIDS cases, different serum lipid levels have been found among different ApoE genotypes. The triglyceride levels in ApoE 3\2 cases are higher than in cases with 3\4 and these cases in turn have higher levels of triglycerides than 3\3 cases. The difference between 3\2 cases and 3\3 cases is wider than the difference between 3\4 and 3\3 (Grunfeld et al, 1997). This is very similar to the findings obtained in the present study for T cell counts. This hypothesis arising from the results presented here requires to be tested, especially because of a growing body of evidence relating to impaired lipid metabolism observed in HIV positive patients receiving reverse transcriptase inhibitors, protease inhibitors or both (Dong et al, 1999; Ledru et al, 2000).

Another possibility although speculative since no evidence has been provided, is that ApoE may directly interact with HIV via the amphipathic α helices of the carboxyl terminus fragment. This region of the ApoE is the lipoprotein-binding domain, while the amino-terminal fragment is where the receptor and proteoglycan binding regions resides. Indirect evidence exists, however, and it is provided by the inhibitory action of the amphipathic α helices of ApoAI on the HIV fusion process *in vitro* (Owens et al, 1990; Martin et al, 1992). One of the physiological actions of the amphipathic α helices of ApoAI is assisting HDL in mediating cholesterol efflux from cells. Both ApoAI and ApoE promote cholesterol efflux from cells and because this process is mediated by the amphipathic α helices of apolipoproteins, similarities in this region between ApoAI and ApoE may almost certainly exist. Thus, cholesterol efflux is differentially mediated by different ApoE isoforms E2 protein being more efficient in promoting cholesterol efflux from cells than any other ApoE isoform. Competition for cholesterol is exerted by the amphipathic α helices leaving fewer of these available for interacting with HIV glycoproteins and the subsequent inhibition of HIV fusion process. This is hypothetical,

because the relevance of these *in vitro* experiments to the *in vivo* setting is uncertain, but nonetheless merits testing.

Other lipids that have been found to be differentially modulated among individuals with different ApoE genotypes include cholesterol, HDL and the levels of ApoB. Cholesterol levels have been found higher in *ε4* carriers than in *ε2* carriers, which makes it more difficult to explain a putative effect of cholesterol levels on T cell homeostasis. The same is applicable to ApoB content on lipoproteins and HDL levels (Dallongeville et al, 1992). However, hypertriglyceridemia, and decreased levels of HDL and LDL have been found in cases with CD4+ T cell counts below 318/μl and significant differences in HDL levels among cases with CD4+ T cell counts above and below 500 cells/μl has also been reported (Zangerle et al, 1994). An association between triglyceride levels in plasma and soluble levels of TNF has been shown and an inverse correlation between HDL and soluble TNF have also been reported (Zangerle et al, 1994). Increased plasma levels of TNFα has also been observed during successful HAART therapy and one of the proposed mechanisms involves the accumulation of TNFα producing T cells (CD4 and CD8). The accumulation of these TNFα producing cells was significantly associated with lipid levels. Thus CD8 positive cells were associated with cholesterol, triglycerides and ApoB and inversely correlated with HDL levels. In contrast, TNFα producing CD4 cells were inversely correlated with triglycerides and ApoB levels and positively correlated with HDL levels (Ledru et al, 2000). Furthermore, CD8 lymphocytosis was observed in patients with lipodystrophy associated with HAART. Cases affected by lipodystrophy have increased levels of cholesterol, triglycerides and ApoB when compared to other individuals in similar treatment regimen without lipodystrophy (Ledru et al, 2000).

The above-mentioned effects of certain lipids on T cell homeostasis can explain the findings obtained in the present study. Although most cases included in this study were not treated with protease inhibitors, which were introduced in Edinburgh after most of the studied cases had died, lipid profile disturbances are observed in AIDS cases. Consequently, the ApoE genotype of individual cases might indirectly contribute to the homeostasis of T cells in AIDS cases due to the effect of ApoE on the metabolism of lipids. It seems likely that many factors acting together, such as cytokine level, plasma viraemia, treatment and probably ApoE genotype as well as systemic factors such as hepatic function may be modulating T cell homeostasis. An association between other cytokines namely IFNγ and hypertriglyceridemia has been reported. In one study of AIDS cases and ApoE abnormalities, a weak correlation was found between ApoE and circulating levels of IFNγ (Grunfeld et al, 1997).

Results from this study also showed that the median values for CD4 and CD8 cell counts for cases homozygous for *ε2* were lower than the median values for other homozygous (3\3, 4\4) cases. Although the number of homozygous cases for *ε2* and *ε4* is very limited, it may be that the identity of other alleles accompanying one *ε2* allele has an impact on T cell homeostasis as it has for lipid metabolism.

ApoE has also been shown to have an influence on the immune system. Immune system abnormalities have been described in ApoE deficient mice (Roselaar and Daugherty, 1998; de Bont et al, 1999). Some *in vitro* studies have shown that delipidated ApoE can inhibit the proliferation of CD4 and CD8 T cells after mitogen stimulation and activation, (Hui and Harmony, 1980; Avila et al, 1982; Pepe and Curtiss, 1986; Kelly et al, 1994). More recent findings have confirmed the same effect on T cells by ApoE E3 and E4 proteins and have shown impairment of delayed type

hypersensitivity responses in ApoE null animal models. This indicates an important role for ApoE as an immunomodulating factor (Laskowitz et al, 2000). Although the mechanism by which ApoE down-modulates the immune response remains obscure, the binding of ApoE to an unidentified lymphocyte cell surface receptor was suggested. LDL receptor present on lymphocytes supports proliferation of lymphocytes after mitogen activation, but it has been shown that the down-regulation of lymphocyte proliferation is independent of that particular receptor (Cuthbert and Lipsky, 1984b). Thus this so-called immunosuppressive receptor seems to be different from LDL receptor, and more likely candidates are the transferrin receptor and HSPG (Reviewed in Mahley and Rall, 2000). An alternative explanation could be that ApoE E2 protein, (which has a defective LDL receptor binding ability) due to the mutation at codon 158 (near the LDL receptor region) fails to stimulate proliferation of mitogen stimulated lymphocytes (cytokine stimulation), preventing in that way a normal immune reaction against HIV infected cells. These explanations remain speculative since no studies have been done in humans and the possibility that a differential effect of the ApoE/LDL receptor between different subsets of T cells (naïve and memory cells) has not been explored.

During the analysis of results, a significant association between ApoE genotype and CD8 counts was obtained for the whole cohort, but very similar Pearson's X^2 values were obtained for drug users. This suggests that 6 cases with very rare ApoE genotypes (4\4 and 2\2) were influencing the result in an important manner. Consequently, the statistical power of these results is limited and the possibility of obtaining similar results by chance alone must be considered.

Going back to the differences in the CD4/CD8 T cell counts between the groups in the present study, analysis of results showed that drug users have a higher number of CD4+ T cells than non-drug users with AIDS. Since the CD4 T cell counts indirectly reflect the immune status of HIV infected patients; a slower decline in CD4 counts may suggest a slower progression of HIV disease. As mentioned before in the Edinburgh AIDS cohort of drug users an increased frequency in HIVE has been reported when compared with AIDS non-drug users. It has also been shown that longer survival after AIDS is significantly associated with HIVE (Soontornniyomkij et al, 1998). This indirect evidence suggests a slower HIV disease progression in this cohort of patients. Recent findings, which will be discussed later, suggest that heroin addicts could have an altered response to HIV-1 infection probably mediated by stimulation of opioid receptors. This is followed by increased production of RANTES, which has been shown to prevent HIV entry into the susceptible cells by competing with HIV co-receptors (Cocchi et al, 1995). Transdeactivation of chemokine receptors after opioid receptor stimulation is another possible mechanism by which heroin addicts may have a protective response to HIV infection.

6.3. Effects of the drugs of abuse

The effect of risk factor, namely drug use will be discussed next. In the present study significantly higher values for CD4 and CD4/CD8 ratios were obtained in drug user cases. Although higher values for CD8 T cell counts were also obtained for that group compared with the non-drug user cases, this was not significant.

In humans, heroin use has been shown to significantly decrease the percentage of CD4+ T cells and CD4/CD8 ratio and significantly increase the percentage and total number of CD8+ T cells in peripheral blood (Govitrapong et al, 1998). The findings of the present study support this view.

Morphine, or its derivatives such as heroin, can exert various effects based on the activation of three main cell surface opioid receptors MOR, KOR and DOR. These receptors have been identified in lymphocytes and macrophages (Bidlack et al, 1992; Chuang et al, 1994; Gaveriaux et al, 1995; Sedqi et al, 1995). It has been shown in murine models that MOR modulated some of the effects of morphine in the immune system (Gaveriax-Ruff et al, 1998).

More recently, *in vitro* models using PBMC from human normal donors and MOR agonists, have shown an increased production of MCP-1, IP10 and RANTES (Wetzel et al, 2000). Moreover cultured cells were treated with MOR agonists before cells were infected with T tropic or M tropic HIV-1. A significant increase in RANTES and IP10 was observed in non-activated PBMC. Upon activation, MOR agonists also produced the same effect on RANTES, but higher doses were required. This mimics what could be happening in drug-users. Viral variants show differential stimulation of chemokine production by activated PBMC. A 13 fold increase of RANTES production was obtained after infection of cultured cells with M-tropic HIV variant (Wetzel et al, 2000). *In vitro* stimulation of HIV infected cells with phytohemagglutinin and MOR and KOR angonists have been shown to inhibit HIV p24 antigen expression (Sharp et al, 2001; Peterson et al, 2001). Similarly KOR has been identified in microglial cells and KOR agonists also suppress HIV-1 expression in microglial cells *in vitro* (Chao et al, 1997). One of the proposed mechanisms is the heterologous dimerization of

chemokine receptors (which also act as HIV co-receptors) also known as transdeactivation, (Peterson et al, 2001; Sharp et al, 2001), since it has been shown to happen with other chemokine receptors after activation of MOR and DOR (Grimm et al, 1998). *In vivo* studies are needed to validate this hypothesis.

Taken together these findings suggest that heroin addicts could have an altered response to HIV-1 infection, due to stimulation of opioid receptors. This could be due to the increased production of RANTES, which has been shown to prevent HIV entry by competing with HIV co-receptors (Cocchi et al, 1995) and the reported suppression of HIV-1 p24 antigen in infected CD4+ T cells. On these basis it is likely that drug users could have a slower HIV-1 disease progression, which may result in a slower decline in CD4+ T cells, which escape immune surveillance mechanisms by failing to express HIV antigens. Other studies have shown this differential HIV disease progression among drug users and men who have sex with men (MSM) cases. Significantly faster HIV disease progression to AIDS and to death has been reported in MSM cases compared with drug users (Pehrson 1998). The increase in percentage and total number of CD8+ T lymphocytes observed in heroin addicts could also slow down the progression of HIV disease, since CTL cells are involved in the host immune response to viral infection. Thus, the findings obtained in the present study with regard to CD4 and CD8 counts might be a consequence of the effect of heroin administration alone. This represents one mechanism by which HIV infected drug users might manifest different infection-related progression than do non-drug users.

In summary, the difference in CD4 and CD4/CD8 ratio among the two main groups studied can be explained by a direct effect of heroin itself and/or by an indirect mechanism involving the production of chemokines by stimulation of opioid receptors

by morphine. Among these, the increased production of RANTES can have an impact on HIV disease progression if the *in vitro* results (Sharp et al, 2001, Peterson et al, 2001) can be applied to the *in vivo* situation.

6.4 Histopathological findings

6.4.1 Effect of drug use on lymphoid infiltration of tissues

Histological examination of HIV positive pre-symptomatic drug users disclosed lymphoid infiltrates surrounding blood vessels within the nervous system in 94.7% of the cases together with a degree of white matter and axonal damage. An inflammatory response in the CNS is the most likely explanation for this. Both drug use and HIV infection can promote inflammation within the CNS and inflammatory mediators have been shown to correlate with white matter abnormalities in all stages of SIV infection (a non-human primate model of HIV infection (Boche et al, 1999)). In pre-symptomatic cases myelin abnormalities have been shown to occur and they correlate with IL 6 and IL 1β mRNA expression in brain tissue of the infected macaques (Boche et al, 1999). The presence of lymphocytes around blood vessels supports previous results (Bell et al, 1993). It has been shown that HIV positive pre-symptomatic drug users have significantly more lymphoid infiltrate both in the subarachnoid space and around blood vessels than control cases (Kibayashi et al, 1996; Tomlinson et al, 1999).

6.4.2 Clinico-pathological correlation

The histological feature which strongly associated with cognitive impairment was HIVE ($p<0.0005$), but this association was present in the drug-using group only. A

significant association between cognitive impairment and risk group was also found ($p<0.02$). That is, a higher proportion of cognitively impaired cases was found among AIDS drug users than non-drug users. In the present study, a significant association between impairment and ApoE $\varepsilon 2$ ($p<0.008$) and a significant negative association between cognitive impairment and ApoE $\varepsilon 3$ ($p<0.01$) were found in the drug using group. In the same group, HIVE was significantly associated with ApoE genotype, probably due to the presence of "rare" ApoE genotypes such 2/2, 4/4 and 2/4 cases, all of them having a degree of cognitive impairment. When specific alleles were analyzed HIVE was associated with ApoE $\varepsilon 2$ ($p<0.005$) and significantly negatively associated with ApoE $\varepsilon 3$ ($p<0.006$). Thus, in the drug-using group a highly significant association between HIVE and cognitive impairment has been shown in this study. This association was not observed in the non-drug users group. This observation may be due to the difference in the number of cases studied in the non-DU group. However, as was shown in this study, HIVE was significantly associated to risk factor ($p<0.05$). That is, HIVE was more frequent in drug users than in non-drug users, in keeping with previous studies in this cohort of AIDS cases (Bell et al, 1996). The present study also showed that drug users have significantly higher CD4 T cell counts and CD8 T cell counts, although the latter did not reach statistical significance. The frequency of the $\varepsilon 2$ allele of ApoE was higher in drug users and this same allele significantly correlated with CD8 T cell counts. This study also showed that non-drug users have a higher frequency of opportunistic conditions. Taking together all these findings, it appears that drug users have a degree of immunocompetence, which prevents the development of opportunistic conditions but is permissive for the development of HIVE and, possibly, cognitive impairment. A significant association between HIVE and cognitive impairment was

previously reported in the Edinburgh cohort (Bell et al, 1998). The association of ApoE *ε2* with cognitive impairment may be due to the effect of this allele in the development of HIVE. It is difficult to support a direct effect of this allele on the cognitive deterioration observed during the course of HIV infection; but it may be acting as a confounding variable in the statistical analysis. The same is applicable to the negative association between ApoE *ε3* and cognitive impairment. However, as will be discussed later, AIDS cases with ApoE 3\3 phenotype have significantly fewer microglial cells in the grey matter of the frontal and temporal hippocampus and it has been shown that the presence of macrophages/microglia in the brain of AIDS cases correlates with HAD (Glass et al, 1995). In the non-drug user group, 22% of the cases had a degree of cognitive impairment and all these cases had some degree of white matter and axonal damage. 66.6% of the non-DU cases with cognitive impairment without HIVE had CMV and the same proportion of these cases also had microglial nodules. These findings suggest an important role of the inflammatory milieu in the CNS in the development of cognitive impairment in keeping with previous studies which suggested that microglial activation was a key factor associated with HAD (Glass et al 1995).

In AIDS cases, a perivascular lymphoid infiltrate was seen in 48.9% of the cases with HIVE, and in 82.4% of the cases without HIVE. This histological feature was negatively associated with HIVE ($p<0.01$), but was associated with higher CD8 and CD4 T cell counts ($p< 0.007$, and $p<0.03$ respectively). The number and functional status of both CD4 and CD8 + cells in drug user cases have been discussed previously. It is likely that a better immune status in drug users favors survival and the development of HAD. Previous studies of the Edinburgh cohort of HIV drug users have shown that most of the lymphoid cells in HIV positive pre-symptomatic cases were

CD8+ T cells (Tomlinson et al, 1999). Since the same histological feature was the most frequently observed in pre-symptomatic cases and in AIDS cases and it was found significantly associated with CD8 and CD4 counts, it is likely that perivascular lymphoid infiltrate requires a degree of CD4 helper function, which, is known to modulate the recruitment of other T lymphocytes into the brain.

Another morphological feature associated with HIVE and cognitive impairment was neuronophagia. Neuronophagia was significantly more frequent in cases with HIVE in the drug users group ($p<0.0005$). The significance of this association is not clear since neuronophagia is not regarded as a specific histological feature of HIVE or any other viral infection although it is frequently seen in the brain of cases with viral encephalitis, after hypoxia, head trauma and in neurodegenerative conditions. Thus, the histological evidence of neuronophagia in HIVE may be explained by end stage hypoxia in these cases.

The analysis of 10 cases of group V disclosed a significant association between neuronophagia and cognitive impairment. This must be interpreted with caution since the likelihood of a spurious association is high especially because group V cases also have a high frequency of CMV encephalitis, a condition in which neuronophagia is frequently seen.

The significant association between microglial nodules and HIVE is not surprising since microglial nodules are regarded as a histological feature of HIVE (Budka et al, 1991).

Other histological findings such as the association between CMV and risk factor support previous findings in the Edinburgh HIV cohort (Bell et al, 1996). The association between CMV and low CD4 counts is well established and has already been

discussed. It has been said that possession of an ApoE ε2 allele increases the risk of HSV encephalitis in immunocompetent individuals (Lin et al, 2001). The frequency of ApoE ε2 allele among 6 cases in which CMV inclusion bearing cells were found was 17.6%, which is exceedingly higher than the frequency of the same allele on the normal Scottish population (Cumming and Robertson 1984). Although in the present study the number of cases is too small and the clinical significance of this histological finding is uncertain.

White matter and axonal pathology are also histological features of HIV infection of the CNS and the significant association between HIVE and axonal balloons is therefore not surprising.

Although the number of cases with lymphoma in the present study was very limited (8 cases), four of them had 3\2 ApoE genotype. Because primary lymphomas of the CNS are associated with Epstein Barr virus infection (reviewed in Ambinder 2001), a possible association between ApoE genotype or a specific ApoE allele (ie. ε2) with this viral related disease merits further investigation. The growing body of evidence linking ApoE with viral conditions has been discussed previously but the exact mechanism by which specific ApoE alleles increase the risk of certain viral infections remains unclear.

A larger sample of ApoE genotyped cases with primary CNS lymphoma may clarify the possible association between ApoE and Epstein Barr infection.

6.5 Cognitive impairment

Cognitive impairment was found significantly associated with HIVE in the drug-using group. The presence of HIVE and HAD in a proportion of cases has also been reported in other studies. It has been suggested that HIVE precedes the development of cognitive impairment for a period of time based on the previously reported findings in which, most cases with cognitive impairment have been shown to have HIVE, while not all patients with HIVE had cognitive impairment (Wiley et al, 1994). Studies involving a larger number of cases (641) have shown a reduced risk of developing cognitive impairment with CD4 counts above 260 cells/μl of blood, suggesting a link between immunodeficiency and HAD (Cornelisse et al, 2000). Conversely, patients with cognitive impairment have been shown to have a significantly increased relative risk of death than patients without cognitive impairment ($p<0.005$) (Ellis et al, 1997b). After adjusting for CD4 counts and other predictors of survival, the relative risk of death for cognitively impaired cases remained significantly increased (Ellis et al, 1997b) suggesting that cognitive impairment is a late event on HIV disease.

Regarding CD68 counts, in almost all cases studied, higher values were obtained in white matter areas than in grey matter areas. Comparisons between AIDS cases and HIV negative cases disclosed significantly greater positivity in all areas studied in AIDS cases.

Two main findings were observed:

The first refers to the number of positive pixels for CD68 among different groups of patients. Increasing number of positive pixels was found when analysis from normal controls was compared to HIV negative individuals, suggesting that drug use on its own

can stimulate microglial proliferation supporting previous findings in the Edinburgh cohort (Tomlinson et al, 1999). This microglial proliferation was more pronounced in the grey matter of the thalamic section in the drug-using group, suggesting that this brain area may be especially susceptible to the effects of drug use.

The HIV positive pre-symptomatic drug user group differed significantly from AIDS cases in the number of CD68 positive macrophage/microglial cells in the grey matter of the temporal hippocampus, suggesting that this anatomical area is a target for advanced HIV infection of the CNS. Since CD68 cells have been shown to produce and express a number of chemokines and their receptors (Sanders et al, 1998) and these inflammatory mediators have been shown to underlie the neurodegenerative process in AIDS, the finding of an increased number of these cells in the temporal hippocampus may support previous observations including decreased number of neuronal sub-populations in the hippocampal areas (Masliah et al, 1992).

In drug users with AIDS, a different pattern of microglial proliferation appeared. Significantly greater positivity for CD68 as compared with pre-symptomatic cases was found in temporal hippocampus and thalamus. It is also interesting that the grey matter of the thalamic section showed significantly higher values in drug users with AIDS than in pre-symptomatic HIV positive drug users. This finding suggests that a possible synergistic effect of HIV infection and drug use is operating in AIDS drug users since macrophage/microglial cell numbers have been shown to correlate with cognitive impairment (Glass et al, 1995).

The second main finding related to the differential values for CD68 positivity among different ApoE genotypes. In all temporal areas (white and grey matter), cases with an ApoE 3\3 genotype had significantly lower values than cases with ApoE genotypes

different from 3\3. Significantly lower values were also obtained for grey matter of frontal lobes. The ε3 allele was associated with the lower CD68 values while the *ε4* allele was associated with higher values. A significant association between CD68 and ApoE genotype was found. No studies relating microglial cell numbers and ApoE genotype in AIDS cases have been reported in the literature. In Alzheimer disease, however, similar results to those found in the present study have been reported (Egensperger et al, 1998). In Alzheimer disease cases *ε4* carriers have increased numbers of activated microglial cells as well as greater areas of tissue covered by these cells. The highest values were found in cases with ApoE genotype 4\4 followed by cases with 3\4 and 3\3 cases in turn had the lowest numbers of activated microglial cells (Egensperger et al, 1998).

These findings suggest that the effect of ApoE on microglial activation is not restricted to Alzheimer's disease, but can also be found in other CNS disorders. Given that the number of cases in this study is limited these findings require further investigation.

END STATEMENT

The findings of the present study suggest a sequence of events as follows: Drug users have a higher frequency of ApoE *ε2* allele. This allele, by still uncertain mechanisms is influencing the rate of progression of HIV infection in these patients, probably by an effect on T cell homeostasis and microglial activation, favoring the development of HIVE and indirectly the establishment of cognitive impairment. This association between ApoE *ε2* allele and HIVE has not been investigated previously but may help to explain the differential presentation of HIVE among AIDS cases. However, caution must be exercised since the number of cases studied although exceeding previous studies is still too low to make dogmatic conclusions. In addition, ApoE *ε2*, which is the rarest allele of the ApoE polymorphisms has often been omitted from previous surveys. A study involving a larger sample of cases will certainly address the role of ApoE *ε2* allele in the development of HIVE.

The findings of the present study and the conclusions arising from it challenge the present view, which is based on the "good and bad" ApoE alleles. It appears that all ApoE alleles have a specific effect, not only in lipid metabolism, but also in the immune system and neurodegeneration. The findings with respect the number and possibly functions of ApoE *ε2* on CD4 and CD8 T cells in the setting of HIV infection merit further study due to the clinical implications of such findings. The lack of a significant association between ApoE phenotype and CD4 counts suggests that the effect of ApoE genotype on T cell homeostasis may be differential. Also the differential effect of ApoE alleles on the number of microglial cells in the central

nervous system merits further investigation because of the association of the inflammatory process and some neurodegenerative conditions. Although the number of cases in which microglial quantitation was carried out in the present study is low, they support previous similar findings in Alzheimer's disease.

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APPENDIX I

Clinical classification systems for HIV infection

WHO		CDC	
Stage I	1) Asymptomatic. 2) Persistent generlized lymphadenopathy. 3) Acute retroviral syndrome. 4) Performance scale level (normal activity.	**Category A**	1) Asymptomatic HIV infection. 2) Persistent generalized lymphadenopathy. 3) Acute HIV infection with accompanying illness or history of acute syndrome.
Stage II (mild disease)	1) Weight loss less than 10%. 2) Minor mucocutaneous manifestations. 3) Herpes Zoster within previous 5 years. 4) Recurrent upper respiratory tract infections. 5) Performance scale level (almost fully ambulatory.	**Category B**	1) Bacillary angiomatosis. 2) Candidiasis oropharingeal. 3) Candidiasis vulvovaginal (persistent frequent or poorly responsive to treatment. 4) Cervical dysplasia (moderate or severe). 5) Constitutional symptoms (chronic fever of diarrhea lasting more than one month 6) Oral hairy leukoplakia. 7) Herpes Zoster (two distinct episodes or more than one dermatome involved). 8) Idiopathic thromboctopenic purpura 9) Listeriosis. 10) Pelvic inflammatory disease. 11) Peripheral neuropathy.
Stage III (moderate disease)	1) Weight loss greater than 10%. 2) Chronic diarrhoea (lasting for more than one month). 3) Prolonged fever (lasting for more than one month. 4) Oral candidiasis. 5) Oral hairy leukoplakia. 6) Pulmonary tuberculosis (developing during the previous year). 7) Severe bacterial infections. 8) Chronic vulvovaginal candidiasis lasting more than one month. 9) Performance scale level (remain in bed less than 50% of the day time).	**Category C**	1) Candidiasis of bronchi, trachea or lungs. 2) Candidiasis of oesophagus. 3) Cervical cancer-invasive. 4) Coccidioidomycosis disseminated or extrapulmonary. 5) Cryptococosis extrapulmonary. 6) Cytomegalovirus infection (other than live, spleen and lymph nodes). 7) Cytomegalovirus retinitis. 8) HIV-related encephalopathy. 9) Chronic herpes simplex ulcer or bronchitis, pneumonitis or oesophagitis. 10) Histoplasmosis disseminated of extrapulmonary. 11) Isosporidiosis, chronic intestinal. 12) Kaposi Sarcoma. 13) Lymphoma Burkitt's. 14) Lymphoma immunoblastic. 15) Lymphoma (primary) of the brain. 16) Mycobacterium avium or kansasii disseminated or extrapulmonary. 17) Other mycobacterium infection disseminated or extrapulmonary. 18) Pneumocystis carinii pneumonia. 19) Recurrent pneumonia. 20) Progressive multifocal leukoencephalopathy. 21) Salmonella septicemia recurrent. 22) Toxoplasmosis of the brain. 23) HIV wasting syndrome.
Stage IV (severe disease)	1) HIV wasting syndrome (weight loss >10% and chronic diarrhea or chronic weakness and unexplained fever. 2) Pneumocystis carinii pneumonia. 3) Toxoplasmosis of the brain. 4) Cryptosporidiosis with diarrhoea lasting more than one month. 5) Isosporidiosis with chronic diarrhea. 6) Extrapulmonary cryptococosis. 7) Cytomegalovirus infection affecting organs other than liver, spleen, and lymph nodes. 8) Visceral or chronic mucocutaneous herpes simplex virus infection. 9) Progressive multifocal leukoencephalopathy 10) Any disseminated endemic mycosis. 11) Candidiasis of the oesophagus, trachea, bronchi or lungs. 12) Disseminated atypical micobacterium infection. 13) Non-typhoidal salmonella septicemia. 14) Extrapulmonary tuberculosis. 15) Lymphoma. 16) Kaposi Sarcoma. 17) HIV-related encephalopathy. 18) Performance scale level (remain in bed >50% day time).		

Source: (Lifson et al, 1995; CDC, 1992).

Appendix II

Cause of death for cases in Groups III, IV and V

Cause of death	Group		
	Group III Pre-symptomatic HIV positive drug users (n=38)	**Group IV** Drug users HIV positive with AIDS (n=84)	**GroupV** Haemophiliacs/MSM with AIDS (n=44)
1	7	37	17
2	4	16	2
3	0	3	9
4	0	4	5
5	1	5	9
6	24	19	2
7	2	0	0
Total	38	84	44

Key:
1: Bronchopneumonia, pneumonia, lung abscess.
2: Systemic failure: pulmonary oedema, acute renal failure, liver failure, other metabolic abnormalities.
3: CNS lymphoma.
4: Other malignancies: Kaposi's sarcoma, systemic lymphoma, Hodgkin lymphoma.
5: Systemic infections including opportunistic (*pneumocystis carinii* pneumonia, atypical *mycobacterium*, cytomegalovirus, toxoplasmosis, septicaemia).
6: Accidental/Suicidal (drowning, hanging, drug overdose).
7: Undetermined following autopsy (suspected drug overdose).

APPENDIX III

Clinical data and neuropathological findings among cases of Group IV

NUMBER	AGE	GENDER	C.I	APOE	HIVE	PVLI	MGN	NPHAG	A B	V.DAM	MINERAL	CMV	LYMPH	PNI
B 059	34	M	+	3 & 3	++	-	++	+	-	-	-	-	+	**
C 074	28	M	-	3 & 2	++	++	++	++	+	-	-	-	-	**
C 102	25	F	**	3 & 3	+	++	+	-	+	-	+	-	-	**
C 329	30	M	++	3 & 4	++	-	++	-	+	-	-	-	-	**
D 434	28	F	-	3 & 4	+	+	+	+	+	+	-	-	-	**
D 457	25	F	+	3 & 3	++	+	++	-	-	+	-	+	-	+
E 021	41	M	++	3 & 4	-	++	+	-	-	-	-	-	-	-
E 079	28	M	+	4 & 4	++	+	+	-	-	+	-	-	-	+
E 127	33	F	-	3 & 3	-	++	+	-	-	-	++	-	-	-
E 156	25	F	++	2 & 2	++	-	+	-	++	-	++	+	-	+
E 1G	33	M	-	3 & 4	+	++	+	+	-	-	-	++	-	+
E 206	31	M	-	3 & 3	-	-	-	-	-	-	-	-	+	+
E 246	33	M	+	3 & 3	++	+	+	+	++	-	-	-	-	+
E 283	28	M	-	3 & 3	+	++	+	-	+	-	-	-	-	+
E 342	36	M	-	3 & 3	-	++	-	-	+	+	-	-	-	+
E 353	26	M	++	3 & 3	++	+	+	-	+	-	+	-	-	-
E 355	31	M	**	3 & 3	-	+	-	-	-	-	+	-	-	+
F 118	33	M	++	3 & 2	++	++	++	-	+	-	-	-	-	+
F 222	25	M	-	3 & 3	-	+	+	-	-	-	-	-	-	+
F 353	34	M	+	3 & 4	++	+	++	+	-	-	+	-	-	+
F 415	45	M	+	3 & 3	++	-	+	-	-	-	-	++	-	-
F 446	25	M	+	3 & 3	++	-	++	++	-	-	-	-	-	+
F 448	37	M	+	3 & 3	-	-	++	-	-	-	-	+	-	+
F 451	46	M	+	3 & 3	-	++	++	-	-	-	-	-	-	+
G 068	30	M	+	3 & 4	-	++	-	-	-	-	-	-	-	-
G 120	27	M	-	3 & 3	-	++	-	-	-	-	-	-	-	-
G 135	35	F	++	3 & 2	++	++	+	+	++	-	-	-	-	-
G 176	33	M	++	3 & 4	++	+	++	+	+	+	+	-	-	-
G 284	34	M	++	3 & 2	++	+	++	++	+	+	-	-	-	+
G 319	28	F	-	3 & 3	-	+	+	-	-	-	-	-	-	+
G 320	30	M	++	3 & 3	++	++	++	++	++	-	-	-	-	+
G 357	28	F	-	3 & 3	-	++	+	++	-	+	-	-	-	+
G 358	28	M	++	3 & 2	++	+	++	++	+	-	-	-	-	-
H 038	28	F	+	2 & 2	++	-	++	++	+	+	-	-	-	+
H 116	31	M	++	3 & 4	+	++	++	-	-	+	-	-	-	+
H 162	34	M	-	3 & 4	-	++	-	-	+	-	-	-	-	+
H 170	39	F	-	3 & 3	-	+	-	-	-	-	-	-	-	-
H 186	41	M	+	3 & 3	+	+	+	+	+	-	-	-	-	+
H 234	28	M	++	3 & 2	++	-	++	-	-	-	-	-	+	+
H 271	34	F	-	3 & 3	-	++	+	-	+	-	-	-	-	+
H 280	27	M	++	3 & 3	+	+	+	+	-	-	-	-	-	-
I 015	30	M	+	4 & 4	++	-	++	++	+	-	+	-	-	+
I 103	30	F	**	3 & 4	-	++	++	-	-	-	-	-	-	+
I 199	32	F	+	3 & 3	+	+	+	+	-	-	-	-	-	+
J 013	35	M	-	3 & 3	-	++	+	-	-	-	-	-	-	+
J 173	34	M	+	3 & 4	++	-	++	++	+	+	-	-	-	+
K 021	48	F	++	2 & 4	+	++	-	-	-	-	+	-	-	**
K 073	32	F	++	3 & 3	++	++	++	+	-	-	+	-	-	**
L 177	40	M	++	3 & 3	-	+	-	-	-	-	+	-	-	**

Key: C. I : Cognitive impairment, HIVE: HIV encephalitis, PVLI: Perivascular lymphocytic infiltrate, MNG: Microglial nodules, NPHAG: Neuronophagia, A B: Axonal Balloons, V DAM: Vascular damage, Mineral: Mineralization, CMV: cytomegalovirus, LYMPH: Lymphoma, PNI: peripheral nerve inflammation, **: not available

Clinical data and neuropathological findings among cases of Group V

NUMBER	AGE	GENDER	C.I	APOE	HIVE	PVMI	MGN	NPHAG	A B	V.DAM	MINERA	CMV	LYMPH	PNI
C 2L	50	M	-	3 & 3	-	+	+	-	+	-	-	+	+	+
E 276	30	M	+	3 & 4	+	+	++	-	++	-	+	++	-	+
E 277	49	M	+	3 & 3	-	-	++	++	++	+	++	++	-	+
E 334	28	M	-	3 & 3	++	-	++	-	-	-	-	-	-	**
E 375	60	M	-	3 & 2	-	+	+	-	-	-	-	-	-	+
F 001	27	M	-	3 & 2	+	-	+	-	-	-	-	+	+	+
F 002	33	M	-	3 & 2	-	-	++	++	+	+	-	++	+	**
F 218	30	M	++	3 & 2	-	-	-	+	+	-	-	++	+	-
F 334	36	M	-	3 & 3	-	+	-	-	-	-	++	+	-	-
G 016	39	M	-	3 & 4	-	++	-	-	-	-	-	-	-	+
G 020	29	M	**	3 & 4	+	+	+	+	++	-	+	-	-	+
G 111	31	M	-	3 & 3	-	++	-	-	-	+	-	-	-	+
G 184	31	M	+++	3 & 2	-	++	++	++	+	-	-	++	-	+
H 276	26	M	-	3 & 3	-	++	-	-	+	-	+	-	-	+
H 313	41	M	-	3 & 3	+	+	++	-	++	-	-	-	-	+
I 0B	22	M	-	3 & 2	+	+	+	-	-	-	-	-	-	+
I 108	51	F	+++	3 & 3	-	+	-	+	+	-	+	-	-	+
I 277	36	M	+++	3 & 2	++	-	++	++	-	-	-	-	-	+
I 2I	34	M	-	3 & 3	-	++	-	-	-	-	+	-	-	+
I 310	47	M	-	4 & 4	-	++	+	+	+	-	-	-	-	+
I 363	40	M	-	3 & 4	-	++	++	+	+	-	-	++	-	+
I 371	49	M	+	3 & 3	+	-	++	-	+	-	-	-	-	+
I 3B	29	M	+	3 & 3	-	+	++	-	+	-	-	+	-	+
J 033	27	M	+	3 & 3	-	-	++	-	+	-	-	-	-	+
J 128	48	M	+	3 & 3	++	-	++	++	+	-	+	+	-	+
J 160	45	M	-	3 & 3	+	-	+	-	+	-	+	-	-	+
J 206	37	M	+	3 & 4	++	-	++	++	-	+	-	+	+	-
K 020	49	M	-	3 & 2	++	+	++	-	+	-	-	-	-	**
K 100	37	M	**	3 & 3	++	+	++	-	-	-	-	-	-	**

Key: C. I : Cognitive impairment, HIVE: HIV encephalitis, PVLI: Perivascular lymphocytic infiltrate, MNG: Microglial nodules, NPHAG: Neuronophagia, A B: Axonal Balloons, V DAM: Vascular damage, Mineral: Mineralization, CMV: cytomegalovirus, LYMPH: Lymphoma, PNI: peripheral nerve inflammation, **: not available

Neuropathological findings by Brain Area among cases of Group IV

NUMBER	T.MGC	T.NPHAG	T.AXB	TA.MGC	TA.NFAG	TA.AXB	NC.MGC	NC.NFAG	NC.AXB	MB.MGC	MB.NFAG	MB.AXB	MD.MCG	MD.NFAG	MD.AXB
B 059	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-
C 074	+	+	-	+	+	-	+	+	-	+	+	+	+	+	-
C 102	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
C 329	+	-	-	**	**	**	+	-	-	+	-	-	**	**	**
D 434	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
D 457	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
E 021	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E 079	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-
E 127	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E 156	+	-	-	+	-	+	+	-	+	+	-	+	-	-	-
E 1G	-	-	-	-	-	-	-	+	-	-	-	-	**	**	**
E 206	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
E 246	+	-	-	+	-	-	+	+	-	**	**	**	+	-	+
E 283	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-
E 342	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
E 353	+	-	-	+	-	+	+	-	+	-	-	-	+	-	-
E 355	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 118	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
F 222	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 353	-	-	-	+	-	-	+	-	-	+	+	-	**	**	**
F 415	-	-	-	+	-	-	+	-	-	+	+	+	-	-	-
F 446	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-
F 448	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F 451	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G 068	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G 120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G 135	-	+	-	+	-	+	+	-	-	-	-	-	-	+	-
G 176	+	-	-	+	+	+	+	-	-	-	-	-	**	**	**
G 284	+	+	-	+	+	+	+	+	-	+	+	+	-	+	-
G 319	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
G 320	+	+	-	+	-	+	+	-	+	+	+	-	+	+	-
G 357	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
G 358	+	+	-	+	-	+	+	+	+	+	+	-	+	+	-
H 038	+	+	-	+	+	+	+	+	-	+	-	-	+	+	-
H 116	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
H 162	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H 170	-	-	-	-	-	-	-	-	-	**	**	**	-	-	-
H 186	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
H 234	+	-	-	+	-	-	+	-	-	-	-	-	**	**	**
H 271	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H 280	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
I 015	+	+	-	+	+	+	+	+	-	+	+	-	-	+	-
I 103	-	-	-	-	-	-	-	-	-	-	-	-	**	**	**
I 199	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
J 013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J 173	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-
K 021	-	-	-	+	-	-	+	-	-	+	-	-	**	**	**
K 073	+	-	-	+	-	-	+	+	-	**	**	**	**	**	**
L 177	-	-	-	-	-	-	-	-	-	-	-	-	**	**	**

Key: T: Temporal, TA: Thalamus, NC: Neocortex, MB: Midbrain, MD: Medulla, MGC: Multinucleated Giant Cells, NPHAG: Neuronophagia, AXB: Axonal balloons. *: not available

Clinical data and neuropathological findings among cases of Group V

NUMBER	AGE	GENDER	C.I	APOE	HIVE	PVMI	MGN	NPHAG	A B	V.DAM	MINERA	CMV	LYMPH	PNI
C 2L	50	M	-	3 & 3	-	+	+	-	+	-	-	+	+	+
E 276	30	M	+	3 & 4	+	+	++	-	++	-	+	++	-	+
E 277	49	M	+	3 & 3	-	-	++	++	++	+	++	++	-	+
E 334	28	M	-	3 & 3	++	-	++	-	-	-	-	-	-	**
E 375	60	M	-	3 & 2	-	+	+	-	-	-	-	-	-	+
F 001	27	M	-	3 & 2	+	-	+	-	-	-	-	+	+	+
F 002	33	M	-	3 & 2	-	-	++	++	+	+	-	++	+	**
F 218	30	M	++	3 & 2	-	-	-	+	+	-	-	++	+	-
F 334	36	M	-	3 & 3	-	+	-	-	-	-	++	+	-	-
G 016	39	M	-	3 & 4	-	++	-	-	-	-	-	-	-	+
G 020	29	M	**	3 & 4	+	+	+	+	++	-	+	-	-	+
G 111	31	M	-	3 & 3	-	++	-	-	-	+	-	-	-	+
G 184	31	M	+++	3 & 2	-	++	++	++	+	-	-	++	-	+
H 276	26	M	-	3 & 3	-	++	-	-	+	-	+	-	-	+
H 313	41	M	-	3 & 3	+	+	++	-	++	-	-	-	-	+
I 0B	22	M	-	3 & 2	+	+	+	-	-	-	-	-	-	+
I 108	51	F	+++	3 & 3	-	+	-	+	+	-	+	-	-	+
I 277	36	M	+++	3 & 2	++	-	++	++	-	-	-	-	-	+
I 2I	34	M	-	3 & 3	-	++	-	-	-	-	+	-	-	+
I 310	47	M	-	4 & 4	-	++	+	+	+	-	-	-	-	+
I 363	40	M	-	3 & 4	-	++	++	+	+	-	-	++	-	+
I 371	49	M	+	3 & 3	+	-	++	-	+	-	-	-	-	+
I 3B	29	M	+	3 & 3	-	+	++	-	+	-	-	+	-	+
J 033	27	M	+	3 & 3	-	-	++	-	+	-	-	-	-	+
J 128	48	M	+	3 & 3	++	-	++	++	+	-	+	+	-	+
J 160	45	M	-	3 & 3	+	-	+	-	+	-	+	-	-	+
J 206	37	M	+	3 & 4	++	-	++	++	-	+	-	+	+	-
K 020	49	M	-	3 & 2	++	+	++	-	+	-	-	-	-	**
K 100	37	M	**	3 & 3	++	+	++	-	-	-	-	-	-	**

Key: C. I : Cognitive impairment, HIVE: HIV encephalitis, PVLI: Perivascular lymphocytic infiltrate, MNG: Microglial nodules, NPHAG: Neuronophagia, A B: Axonal Balloons, V DAM: Vascular damage, Mineral: Mineralization, CMV: cytomegalovirus, LYMPH: Lymphoma, PNI: peripheral nerve inflammation, **: not available

APPENDIX IV

Mean and median values for CD68 counts by area and group

Group	Total Number of positive pixels	**Frontal**		**Temporal**		**Thalamus**	
		Grey matter	White matter	Grey matter	White matter	Grey matter	White matter
Group I	**Mean**	**424.5**	**775.7**	**360.6**	**997.1**	**271.2**	**356.5**
N=4	Median	293.3	627.8	399.5	975.3	221.8	317.0
Group II	**Mean**	**406.6**	**1231.2**	**566.4**	**1202.3**	**463.8**	**601.6**
N=10	Median	351.6	1233.1	511.0	1183.5	402.7	528.0
Group III	**Mean**	**681.6**	**1732.6**	**363.3**	**1498.3**	**631.3**	**790.0**
N=7	Median	768.3	1724.7	399.6	1191.8	478.2	851.2
Group IV	**Mean**	**1804.6**	**4251.2**	**2276.4**	**4625.4**	**3175.4**	**2408.1**
N=9	Median	1545.8	3842.7	1760.1	4543.0	2324.5	2266.4
Group V	**Mean**	**1885.4**	**4264.1**	**1543.1**	**4855.7**	**1844.9**	**1875.2**
N=6	Median	1745.7	3940.0	1377.6	3941.1	1426.3	1743.5